DISSERTATION

BIOLOGY AND MANAGEMENT OF BLACKLEG DISEASE OF POTATO CAUSED BY *DICKEYA DIANTHICOLA* (ME23)

Submitted by

Shaista Karim

Department of Agricultural Biology

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2021

Doctoral Committee:

Advisor: Amy Charkowski
Co-Advisor: Pankaj Trivedi

Courtney Jahn
Mark Uchanski

ABSTRACT

BIOLOGY AND MANAGEMENT OF BLACKLEG DISEASE OF POTATO CAUSED BY *DICKEYA DIANTHICOLA* (ME23)

Potato is the most commonly consumed vegetable in the United States, where people each an average of 49.2 pounds per person per year. About 80% of potatoes in the US are produced in Idaho, followed by Washington, Wisconsin, and Oregon. Potato is a vegetatively propagated crop, and progeny tubers serve as seed for cultivation the following year. Therefore, tuber-borne pathogens, such as bacteria that cause blackleg, result in serious economic losses when progeny tubers are contaminated by pathogens. Blackleg of potato is characterized by blackening of the basal part of stem and rotting of seed tubers. It is caused by *Pectobacterium* and *Dickeya* species, which are in the Pectobacteriaceae family and are collectively referred to as the soft rot *Pectobacteriaceae* (SRP).

In 2015, multiples outbreaks of blackleg and soft rot occurred in Northeastern United Sates. This outbreak of the disease also impacted potato production all across the neighboring states, as well as other northeastern and mid-Atlantic states where Maine seed potatoes were shipped. It is most likely that prior to the *Dickeya dianthicola* (ME23) outbreak in the US in 2015, *Dickeya* was present in seed potatoes and farms in the affected states for at least a few years. However, rain in 2013 and 2014 spread the pathogen and cool temperatures caused the bacterium to remain latent in the tubers. Warm temperature in 2015 on commercial farms that used this *Dickeya*-infested seed resulted in significant outbreaks.

The fields with outbreaks had no previous history of blackleg, the pathogen was suspected to have been present somewhere in the environment, then multiplied suddenly in response to favorable conditions, such as a heavy rain with subsequent surface pooling, and thus caused an outbreak. To prevent further spread of the disease, the primary infection source and the route of infection of the blackleg pathogen must be identified. Being able to discriminate exact subspecies of *Dickeya* from the others could help reduce the infection and to understand the epidemiology of the pathogen. Therefore, my research focused on development of reliable and accessible detecting tools for *D. dianthicola* (ME23).

Unfortunately, many commercial potato varieties are susceptible to the diseases caused by SRP. Very few are tolerant, and production is compromised due to infection caused by *D. dianthicola* and high risk of spreading bacteria in other farms if potato seeds are infected. This led to an urgent need to screen for resistance against blackleg disease. There is insufficient information available for potato breeders on relative resistance or tolerance of commercial potato varieties to *Dickeya* and *Pectobacterium* spp. For the purpose of our work with SRP, we use the term resistance for plants that remain asymptomatic, or nearly so, after inoculation with *Dickeya* or *Pectobacterium* in typical temperature, humidity, and oxygen-level conditions.

In addition, there is almost zero evidence of single gene resistance against this pathogen. Rather, disease resistance is quantitative and multigenetic, making it difficult for plant breeders to select for resistance. In addition, blackleg development is highly sensitive to multiple environmental factors including, plant age, availability of favorable environmental conditions and other bacterial pathogen present in the environment, making it difficult to screen varieties for resistance. The molecular and biochemical mechanisms underlying these quantitative resistances

are also poorly understood. Therefore, are not efficiently utilized in potato breeding programs, altogether this makes it difficult to achieve true blackleg disease resistance.

Nevertheless, it has been previously reported that plant resistant relies on production of small molecules such as phytoalexins or phytoanticipins associated with core resistant pathways. For example, these pathways may induce plant hormones associated with resistance, or antimicrobial peptides or enhance cell wall modifications as a physical barrier against plant pathogens. Interestingly, some accessions of the wild diploid species of potato (*Solanum chacoense)* are resistant to blackleg and soft rot diseases caused by SRP. My research focuses on identification of resistant lines of wild diploid potato relatives using physiological, biochemical and metabolic profile.

In my work, I found that the metabolic profile of resistant stem extracts of *S. chacoense* consists of small molecules including phenolics, alkaloids, lipids, amino acids and organic acids, some of which may play a significant role in antimicrobial and anti-virulence properties. I found that the biochemical assays including quorum sensing (QS) and plant cell wall degrading enzymes (PCWDE) correlated with metabolites identified in metabolic profile of resistant accessions. Hence, these assays can be used as a less time consuming and easy tool for screening resistant lines against SRP.

From these findings, I hypothesize that QS inhibiting molecules are responsible for triggering resistance against blackleg in *S. chacoense* and can be used as a potential tool in future breeding programs to achieve maximum resistance in our commercially grown potato varieties.

ACKNOWLEDGEMENTS

Foremost, I would like to express my sincere gratitude to my advisor Dr. Amy Charkowski. Dear Dr. Amy Charkowski, I could have never completed this milestone without your unconditional and continuous support and guidance you have provided me over the last few years. You provided me the freedom to design my research projects without any objection. You have your own way of training your students which I found to be the best way. In our discussions about our research projects, you used to ask me questions which would lead me to the answer. It helped me to critically think about my research and become an independent researcher. Thank you so much for teaching me molecular plant pathology. I also thank you for sponsoring me to attend research meetings which broadened my scientific concepts and understanding. Your support was not limited to lab work, but you also supported and guided me in all tough situations of my life. For that, I will forever be thankful and indebted to you. We had a great relationship, and it has been a blessing to have you as my advisor. Thank you for believing in me when I was not well suited for molecular plant pathology in terms of scientific knowledge. Thank you for providing me opportunity and equip me with tools to make me see this wonderful day of my life.

I am also grateful to Dr. Pankaj Trivedi, for guiding me in my research work. Dr. Trivedi, you always found a way to appreciate and encourage me. I always felt motivated after talking to you about my research. Thank you for always being accessible and happy to help whenever needed. During my prelim exams and committee meetings I used to spend a lot of time studying to prepare for answering your questions. Last but least, thank you for inviting me over on religious eve's when I was thousands of miles away from my family and feeding me traditional delicious meals.

I would also express my gratitude to my committee members, Dr. Courtney Jahn and Dr. Mark Uchanski, for their sincere and valuable guidance and encouragement extended to me.

I am thankful to Dr. Alejandra Huerta for her help and training me on my first day at CSU by teaching me the basic plant pathology tool “pipette” which I have never seen before. Alejandra, you are a great person and a very valuable friend. You were like a family to me at CSU and I will always be thankful for that.

I also want to express my sincere gratitude to my mentors in the lab, Dr. Janak Joshi and Dr. Yuan Zeng and Afnan Shazwan. Thank you for teaching me and motivating me to conduct wonderful research. PhD was a long and dull journey at the same time for me, but whenever I looked up at you, I found myself motivated to conduct science. At many instances, I was able to do my work just because you were around to help me with things.

A special thanks and shout out to dear friend Dr. Ali Asghar. You were a valuable asset that I had at CSU. Your teaching was not only limited to science, but you also made me laugh when I couldn’t smile, taught me communication skills, and of course made me a chef as well. Thank you once again for being there when I needed the most and I wish you good luck with your future endeavors.

I would like to thank all Agricultural Biology faculty, staff and students. My special thanks to Dill Janet and Paul. Thank you for all the hard work to help me meeting official deadline and keeping up with my plants in greenhouse.

I am very grateful to the University of Agriculture Faisalabad for sponsoring part of my studies through “50 overseas Punjab government funding”.

Last but not least, I am thankful to my family and friends. Thank you for loving me so much. I am happy that technology (Skype and WhatsApp) made it possible for me to see you and talk to you almost every day. I love you all.

DEDICATION

I dedicate this dissertation to my sister and my friend; Dr. Ana Cristina Fulladolsa. Dear Ana Cristina, I learned an important lesson of life after meeting with you that family is not about blood, it's all about who is willing be there when you needed the most. You have, and you will always be a role model for me. You taught me determination, truthfulness and honesty. Thank you for being there with me always and being a valuable support. I am sad that I will be leaving country soon and will not be able to spend time with you, but you promised me to visit Pakistan one day. Thank you for making the hardest journey of life smooth and loved. You are the best sister one can ever have from another mother.

TABLE OF CONTENTS

ABSTRACT ii
ACKNOWLEDGEMENTS v
DEDICATION viii
LIST OF TABLES xii
LIST OF FIGURES xiii
CHAPTER I: ROLE OF SPECIALIZED METABOLITES IN PLANT DEFENSE RESPONSES AGAINST BACTERIAL PATHOGENS 1
Synopsis 1
1. Introduction 2
1.1. Phenolic acids – metabolites with multiple roles in plant biology 2
1.2. Alkaloids, a bitter plant toxin with important roles in plant defense 4
1.3. Terpenes, aromatic plant compounds that contribute to plant defense 5
1.4. Fatty acids and derivatives 7
2. Quorum sensing: master regulator of virulence factors in plant pathogenic bacteria 7
3. Effects of plant-derived molecules on bacterial virulence factors 8
3.1. Effects on QS signaling molecules 8
3.2. Anti-biofilm activity of plant derived molecules 13
3.3. Role of plant derived molecules on motility 15
3.4. Plant cell wall degrading enzymes, effector proteins and plant secretion system 17
3.5. Pigments, toxins and other virulence factors 19
4. Conclusions and future prospective 21
REFERENCES 37
CHAPTER II: DEVELOPMENT OF AUTOMATED PRIMER DESIGN WORKFLOW UNIQPRIMER AND DIAGNOSTIC PRIMERS FOR THE BROAD HOST RANGE PLANT PATHOGEN *DICKEYA DIANTHICOLA*[12] 65
Synopsis 65
1. Introduction 65
2. Materials and Methods 69
2.1. Uniqprimer design and implementation on Rice Galaxy 69
2.2. Primer design and in silico analyses 70
2.3. Source and identity of isolates and environmental samples 70
2.4. Bacterial DNA extraction 71

2.5. Specificity of cPCR and qPCR assays 71
2.6. Sensitivity of cPCR and qPCR assays 72
3. Results 72
3.1. Description of the Uniqprimer Rice Galaxy Tool 72
3.2. Design of *D. dianthicola* species-specific primers with Uniqprimer 73
3.3. Sensitivity and specificity of cPCR and qPCR assays 74
3.4. Detection limit of cPCR and qPCR assays 75
3.5. Field sample analysis 75
3.6. Comparison DDI-1 and DDI-2 to extant tools for *D. dianthicola* detection 75
4. Discussion 76
REFERENCES 97
CHAPTER III: IMPROVED DETECTION OF THE POTATO PATHOGEN *CLAVIBACTER MICHIGANENSIS* SUBSP. *SEPEDONICUS* USING DROPLET DIGITAL PCR 103
Synopsis 103
1. Introduction 104
2. Materials and Methods 106
2.1. Bacterial cultures 106
2.2. Tuber core sample preparation 106
2.3. DNA extraction 108
2.4. ELISA and RT-PCR assays 108
2.5. ddPCR assays 109
2.6. False positive rate and limit of detection for ddPCR. 110
3. Results 111
3.1 ddPCR sensitivity in detection of purified Cms DNA 111
3.2. Use of ddPCR to detect Cms in low-titer field samples 112
3.3. Cms is detectable in bulk samples of up to 800 potato cores 112
3.4. Detection of Cms in commercial potatoes using ddPCR 113
3.5. False positive rate 113
4. Discussion 113
REFERENCES 125
CHAPTER IV: FROM METABOLOMICS TO FUNCTIONAL PHENOTYPES: REVEALING METABOLIC FEATURES OF DIPLOID POTATO SPECIES (*SOLANUM CHACOENSE*) IN RESPONSE TO BLACKLEG DISEASE 131
Synopsis 131
1. Introduction 132

2. Materials and Methods 135
2.1. Plant propagation and maintenance 135
2.2. Time-lapse video assay 135
2.3. Stem inoculation for lesion length measurements 136
2.4. Sample preparation for metabolic profiling 136
2.5. Liquid chromatography-mass spectrometry (LC-MS) 137
2.6. Data processing and metabolite annotation 138
2.7. Biochemical assays 138
2.8. Microscopy assays 139
2.9. Statistical analysis 139
3. Results 140
3.1. Virulence assay to rate *Dickeya* infection to *S. chacoense* lines 140
3.2. Lesion length and time-lapse video revealed that wilting symptoms develop faster in susceptible lines than in resistant lines 140
3.3. Metabolite extracts from resistant wild diploid potato lines reduced *D. dianthicola* exoenzyme activity and AHL synthesis 141
3.4. Metabolomic analysis of *S. chacoense* stem extracts using liquid chromatography mass spectrometry 142
3.5. Induction of *S. chacoense* metabolites after inoculation with *D. dianthicola*. 143
4. Discussion 144
REFERENCES 165
CHAPTER V: SUMMARY 180

LIST OF TABLES

CHAPTER I
Table 1.1. Summary of plant derived compounds displaying anti-virulence activity….....24
Table 1.2. Phenolic compounds and their activity against phytopathogens.....29
Table 1.3. Alkaloids and their role as phytopathogens…….....31
Table 1.4. Role of terpenes extracted from essential oil extract against plant pathogen adapted from (Mehnaz et al. 2019) …….....33
Table 1.5. Effects of fatty acids as anti-virulence agents against plant pathogen adapted from (Kumar et al. 2020) …….....35

CHAPTER II
Table 2.1. Strains and genomes used in this study.....87
Table 2.2. Uniqprimer output of candidate *Dickeya dianthicola* diagnostic primers….....93
Table 2.3. Comparison of PCR detection results using *Dickeya dianthicola*- specific and *Dickeya*-general diagnostic primers …….....95

CHAPTER III
Table 3.1. Bacterial isolates used in this study…….....121
Table 3.2. Primer and probe used in this study…….....122
Table 3.3. Comparison of Cms detection methods with asymptomatic tubers…….....123
Table 3.4. Grocery store samples…….....124

CHAPTER IV
Table 4.1. Potato stem metabolites associated with inhibiting virulence of *D. dianthicola* ME23…….....154
Table 4.2. List of metabolites consistent in resistant lines. (A) Before infection (B) after infection …….....160

LIST OF FIGURES

CHAPTER I
Figure 1.1. Bacterial virulence factors addressed in this review as targets for anti- virulence agents…….....…….....23

CHAPTER II
Figure 2.1. Flowchart of the Uniqprimer process for primer design…….....80
Figure 2.2. Whole-genome phylogeny of *Pectobacteriaceae* and control strains used for Uniqprimer *D. dianthicola*-specific detection primer design..... 81
Figure 2.3. Sensitivity and specificity of primers detecting *D. dianthicola* determined through conventional polymerase chain reaction…….....82
Figure 2.4. Detection of *Dickeya dianthicola* (Ddi) in potato field samples from across the US…….....83
Figure 2.5. Primer efficiencies calculated for quantitative polymerase chain reaction *Dickeya dianthicola* detection primers…….....84
Figure 2.6. Detection of *Dickeya dianthicola* isolates ME23 and TXG3 using quantitative polymerase chain reaction (qPCR)…….....…….....85
Figure 2.7. In silico analysis of Pectobacteriaceae diagnostic primers and diagnostic primers and predicted targets….....…….....86

CHAPTER III
Figure 3.1 The primer/probe sets for Cms72 and CelA are sensitive and specific…….....119
Figure 3.2. Cms is detectable in individual and bulk samples of potatoes…….....120

CHAPTER IV
Figure 4.1. Virulence assay to rate *D. dianthicola* infection to *S. chacoense* lines…….....148
Figure 4.2. Effects of resistant and susceptible lines stem extract on exoenzyme activity of *D. dianthicola* ME23…….....…….....149
Figure 4.3. Effects of resistant and susceptible lines extract on N-acyl homoserine lactone (AHL) of *D. dianthicola* ME23…….....…….....150
Figure 4.4. Effect of resistant and susceptible stem extracts on morphology *of D. dianthicola* ME23….....…….....151
Figure 4.5. Metabolic response over time to *D. dianthicola* ME23 infection in resistant and susceptible potato lines…….....…….....152
Figure 4.6. Metabolite response over time to *D. dianthicola* infection and control in stem.....153 of potato lines…….....154

CHAPTER I: ROLE OF SPECIALIZED METABOLITES IN PLANT DEFENSE RESPONSES AGAINST BACTERIAL PATHOGENS

Synopsis

Crop losses due to pathogen attack and pest are a major problem worldwide. Plant resistance is the best defense against diseases and specialized plant metabolites contribute to plant resistance. Plant resistance is especially important for management of bacterial pathogens since there are few pesticides available for bacterial diseases of plants. Defense metabolites present prior to pathogen attack are called phytoanticipins and those produced in response to pathogens are called phytoalexins. In both cases, these metabolites are low molecular weight compounds with unique chemical structures and activities (Pedras et al. 2011; Hammerschmidt 1999; Ahuja et al. 2012). Most work to date with antimicrobial metabolites focuses on human pathogens, so we know far more about how plant metabolites affect virulence of animal pathogens than plant pathogens. The similarities between plant and animal pathogens mean that this work can still inform research in plant pathology. Phytoalexins were first discovered over 70 years ago in experiments with the potato pathogen, *Phytophthora infestans*, inoculated onto an incompatible host plant. Based on their results, the researchers hypothesized that potato tuber cells produce phytoalexins in response to an incompatible *Phytophthora* strain and that these phytoalexins protect the tuber from other compatible races of the pathogen (Pedras et al. 2011). Since then, scientists have investigated the role of phytoalexins in plant-microbe interactions and defense mechanisms against multiple types of pathogens (Holland and O'Keefe 2010; Yang et al. 2009; Jahangir et al. 2009; Boue et al. 2009). Phytoanticipins were discovered in the 1940 and are low molecular weight antimicrobial compounds present in plants before pathogen infection (VanEtten et al. 1994). For example, the

saponins in potato tubers that protect plants against microbes and insects, are a well-known example of a phytoanticipins (Osbourn 2003).

1. Introduction

Plants are in continuous interaction with microorganisms in their natural environment. Some interactions harm plants and trigger their defense reaction, other are beneficial for plants survival. Therefore, interaction between plant and microbes is critical for plant fitness. The defense mechanisms plants developed against microbial pathogens rely, to a large extent, on an enormous variety of plant derived compounds, such as phenolics, alkaloids, terpenes and fatty acids. Plants produce vast number of these bioactive molecules and various roles in plant defense against pathogens are attributed to these molecules. Advances in genome sequencing and metabolomics, as well as software that simplifies analysis of large datasets, have led to a resurgence in interest in these plants derived molecules and their role in inhibition of bacterial virulence factors, including bacterial biofilms, enzymes, motility, toxins and quorum sensing (Fig. 1).

1.1. Phenolic acids – metabolites with multiple roles in plant biology

Phenolic acids are a type of aromatic compounds that contain a phenol ring and organic carboxylic acid (Table. 1). They are found in variety of plant organs, including seeds, fruit periderm, and leaves (Table. 2). Usually, phenolic acids are present in a bound form, for example amides, esters, or glycosides (Pereira et al. 2009). Phenolic acid and their derivatives have diverse structure and are produced by at least four pathways in plants, including as products of the shikimic acid pathway or the phenylpropanoid pathway, as byproducts of the monolignol pathway, or as breakdown products of lignin and other plant cell wall polymers (Mandal et al. 2010). Even though the complete role of phenolic acids in plants remain unknown, they are known to control or participate in diverse functions in addition to plant defense, such as nutrient uptake, enzyme

activity, protein synthesis, photosynthesis, and allelopathy (Lyu et al. 1990; Kiokias et al. 2020). They also have multifunctional roles in plant-microbe interactions outside of the plant structure. For example, they are released into the rhizosphere, where they may repel, interfere with development of, or kill microorganisms (Martens 2002; Bhattacharya et al., 2010).

Quantitative trait loci and individual genes required for phenolic acid synthesis have been identified through biochemical analysis, genome sequencing and mapping, and genome synteny studies (Comino et al., 2007; Niggeweg et al., 2004; Gramazio et al., 2014; Morrell et al., 2011). Given the importance of phenolic acids, developing new plant varieties with increased phenolic acid content is of the utmost importance and has become a focus among many researchers in vegetables such as tomato, pepper, cucumber and other crops (Kaushik et al., 2015). This is challenging, however, since these multiple genes contribute to production of phenolic acids, which complicates plant breeding, and new varieties must still produce food that meets market requirements, including taste and yield.

Plant phenolic acids act as potent quorum sensing (QS) inhibitors and two component system inhibitors, thereby interfering with bacterial gene regulation required for expression of virulence genes (Rutherford and Bassler 2010). Plant phenolics also are efflux pump inhibitors, which may inhibit bacterial resistance to plant antimicrobials (Sharma et al. 2019). In all cases, multiple phenolics produced by plants have these inhibitory activities. The relative activity of the different phenolics, whether they have synergistic effects, and efficient breeding strategies for increasing phenolics that act as phytoanticipins or phytoalexins in crops or ornamental plants are all important areas for future work.

1.2. Alkaloids, a bitter plant toxin with important roles in plant defense

Alkaloids are low molecular weight nitrogen-containing compounds that play important roles in plant defense mechanisms against pathogens. These compounds can be categorized into different classes according to their precursor, such as pyrrolidine, tropane, piperidine, pyridine, quinolizidine, and indoles (Yang and Stöckigt 2010). Plants containing alkaloids has improves defense responses against biotic and abiotic stresses (Table. 4). Unfortunately, the significant benefits of alkaloids in plant defense mechanism are not widely explored or used in crop production because many plant alkaloids are both bitter and toxic to human and animals, such as hepatotix pyrrolizine, indolidine, piperidine and tropane (Matsuura and Fett-Neto 2013; Cortinovis and Caloni 2015; Diaz 2015; Vilariño and Ravetta 2008).

Wild relatives of several major agriculture crops contain toxic alkaloids that may contribute to disease resistance. For example, the tubers of wild potato contain toxic glycoalkaloids such as *a*-chaconine and *a*-solanine, which are responsible for acute toxicity and bitter flavor (Zarin and Kruma 2017). Although they may not be useful in all aspects of crop protection, alkaloids could be used to protect ornamental plants or they could be included in a strategic manner to protect other parts of plants, for example, the leaves, tuber and root and or the roots and leaves of fruit crops.

In many cases, alkaloids inhibit bacteria virulence without affecting the growth and viability of bacteria (Joshi et al. 2020). In the majority of cases, it appears that alkaloids interfere with QS related molecules/ genes to attenuate diseases. This may provide resources to overcome the problems by targeting bacterial virulence factors such as biofilm production or QS, however, the mechanism behind not being able to kill bacteria or limit the growth of bacteria in many cases is still unexplored. In order to efficiently utilize alkaloids in breeding programs, researchers need

to address questions such as: Are bacteria able to infect the progeny or other closely related crops after being exposed to alkaloids? What types of genes are involved in making bacteria survive in highly toxic environment? There is risk associated with alkaloids because of their toxic nature, multiple screening tests must be done before implying them in breeding program and therefore to achieve anti-virulence properties of alkaloids in commercially grown vegetables, which are threaten by many pathogens.

1.3. Terpenes, aromatic plant compounds that contribute to plant defense

Plants produce volatile compounds, such as terpenes, that have significant role in interaction with their environment and that help give each plant species its distinctive odor and taste. The physiochemical properties of terpenes including aroma, reactivity, toxicity and volatility aid in diverse protective functions against biotic and abiotic stresses in plants (Holopainen 2004). Terpenoids (Isoterpenes) are the most diverse and largest group of plant volatile compounds (Pichersky and Gershenzon 2002; Rodríguez-Concepción 2006). Essential oils are the major constituency of terpenes, which are complex hydrophobic compounds containing multiple low molecular weight compounds. They have useful antimicrobial activities against many plant pathogenic bacteria (Amaya et al. 2012; Aoki et al. 2010; Joshi et al. 2016).

In plants, terpenes are produced through the cytosolic mevalonate pathway (MVA) and plastid localized 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (Vranová et al. 2013; Kirby et al. 2015;). Terpenes are highly diverse and over 50,000 unique terpenoids have been discovered in plants so far. They play a key role in plant defense mechanisms against insects, herbivores, bacteria and pathogenetic fungi (Franceschi et al. 2005; Table 5). However, in order to be effective against pathogens, sufficient amount of accumulation prior to invasion of the pathogen is necessary; but these levels might be phytotoxic. Therefore, plants evolved to minimize this toxicity

by sequestering terpenes during differentiation of tissues in extracellular spaces (Fahn 1997, 1988; Doehlemann and Hemetsberger 2013). Many plants accumulate terpenes by differentiating them into trichome, oil glands and resin ducts (Glas et al. 2012; Huchelmann et al. 2017; Mewalal et al. 2017; Zulak and Bohlmann 2010). These tissues are hence dedicated in storage and synthesis of terpenes in plants.

Advances in molecular genetics have provided tools to better understanding genome complexity and thereby, equipped plant breeders to effectively use molecular genetic screening approaches to select the best breeding candidates. Unlike alkaloids, terpenoids are commonly used as natural flavoring compounds in food industries and they tend not to be toxic to animals. Given the importance of terpenes, researchers are now focusing on improving terpene contents in vegetables through breeding programs to enhance plant defense responses against stresses including biotic and abiotic stress (Cebolla-Cornejo et al. 2013). Effective treatment and management against plant pathogens are among the main priorities of plant pathologist. Terpene derivatives are an important and promising source against novel phytopathogens.

Despite many discoveries about anti-virulent role of terpenes in plant pathogens, the ongoing chemical investigation using "omics" tools will continue adding novel information to the field of new discoveries. The highly toxic nature of terpenes to bacteria pathogens by disturbing cell wall integrity and eventually leads to cell lysis is troublesome in some way; however, it is unexplored under what concentration of terpenes might be toxic to animals and humans. Nevertheless, further research on the role of terpenes in plant metabolism and induction signals of terpene synthesis will facilitate to manipulate biosynthetic pathways for improvement of agronomical traits, plant defense against pest and pathogen, hence discovery of novel phytocompounds.

1.4. Fatty acids and derivatives

Fatty acids are major component of lipids in microorganisms, plant and animals. They compose of large straight chain of even numbers of carbon with hydrogen atom at one side of the chain and carboxylic groups on other. The most abundant types of fatty acids are derived from glycerolipid biosynthetic pathway. Fatty acids are well known for their significant functions in basal and systemic plant immunity (Kachroo and Kachroo 2009, Table. 6). Therefore, fatty acids and their dedicated role against phytopathogens have been investigated to develop promising antibacterial compounds (Kachroo and Kachroo 2009; Huang and Ebersole 2010; Sethupathy et al. 2017). The most well-known mechanism of fatty acids against bacteria is the disruption of cell membrane and leakage of intracellular metabolites results in cell lysis (Desbois and Smith 2010; Supardy et al. 2019; Kim et al. 2019). Fatty acids also reduce energy production by interfering electron transport chain system and hence block the nutrient uptake by pathogen and starve them to death (Desbois and Smith 2010).

Like other metabolites, fatty acids provide a protective role through attenuating major virulence factors, including AHL modulating QS and biofilm formation. Recent advances in omics tools have uncovered several targets of fatty acids. However, the initial cues for signal induction and molecular interaction of these fatty acids are still unexplored. Further identification of FAs and more detailed information about their exact mode of action could aid in pathogen management.

2. Quorum sensing: master regulator of virulence factors in plant pathogenic bacteria

Quorum sensing (QS) is a process of cell-to-cell communication that enable bacteria to share information about the cell density in a given population and express their genes accordingly (Rutherford and Bassler 2012). Interestingly, Gram-negative and Gram-positive bacteria utilize unique types of QS mechanisms. Gram-positive bacteria produce autoinducer peptides as a

signaling molecule, while Gram-negative bacteria use small molecules called autoinducers to communicate (Rutherford and Bassler 2012; Wei et al. 2011). In addition to sensing and responding to neighboring bacterial cells, both Gram-negative and Gram-positive bacteria form a multicellular surface bounded aggregates, or biofilms (Hall-Stoodley et al. 2004; Davey and O'toole 2000), which help bacteria to resist challenges from predators, antibiotics and host-immunity (Hall-Stoodley et al. 2004; Donlan and Costerton 2002). Although phytoalexins may interfere with virulence in multiple ways, the main mechanism explored is QS inhibition and QS may account for the majority of the other phenotypes observed. For example, QS can regulate motility, biofilm formation, and toxin and virulence enzyme production (Davies et al. 1998; Parsek and Greenberg 2005; Singh et al. 2000; Joshi et al. 2016). The proteins required for QS in the Gram-negative bacteria acyl-homoserine lactone-based QS system appear to be frequently horizontally transferred, suggesting that bacteria are under external pressure from hosts to escape suppression of QS systems (Joshi 2021 review).

3. Effects of plant-derived molecules on bacterial virulence factors

3.1. Effects on QS signaling molecules

AHL-based QS in bacteria is a relatively simple system. A homoserine lactone synthase (LasI/ExpI family) is needed for production of AHL from the precursors s-adenosyl-L-methionine and a fatty acid by LuxI and their signal is perceived when binding to LuxR, which is a cytoplasmic transcriptional regulator (Fuqua et al. 2001). The AHL can transverse bacterial membranes and once the local concentration is high enough, it binds to a regulatory protein (LasR/ExpR family) and the ability of regulatory protein to bind to DNA changes once bound to AHL. These systems are autoinducible, meaning that the regulatory protein up-regulates AHL production once bound to AHL, resulting in swift regulatory and cell development changes once the system is triggered.

In plant pathogens, AHL-based QS is considered the key regulator that shifts bacterial cells from a saprophytic or stealth mode to a pathogenic mode.

Multiple phenolic acids interfere with bacterial QS and other major virulence factors (Table. 3). Phenolics differ from antibiotics in that they can inhibit specific bacterial activities without inhibiting growth. One of the most exciting recent discoveries in this area is that the plant defense hormone, salicylic acid (SA), directly binds to and inhibits the *Pectobacterium* AHL production (Joshi et al. 2020). Salicylic acid derivatives such as methyl salicylate and salicylamide reduce protease activity in *P. aeruginosa* and this could also occur through QS inhibition (Hu et al. 2013; Kumar et al. 2013; Amalaradjou et al. 2010).

Many well-known phenolic acids have comparable effects on AHL production and QS, but unlike SA and *expI* (Joshi et al. 2020), the binding mechanism remains unknown. For example, curcumin, a polyphenol found in turmeric, targets multiple signaling molecules when tested against *Pseudomonas aeruginosa* (PAO1), an opportunistic pathogen (Gupta et al. 2013). Curcumin attenuates PAO1 virulence by down-regulating QS initiation genes in *P. aeruginosa* infections in both animals and plants (Rudrappa and Bais 2008). Other similar examples include glycosylated flavanones in orange extract, ellagic acid in pomegranate extract, cinnamaldehyde, rutin and resveratrol, which were tested under their minimum inhibitory concentration against plant and animal pathogens. These chemicals reduced AHL production in both *Yersinia enterocolitica* and *Pectobacterium carotovorum* (Truchado et al. 2012a; Truchado et al. 2012b). A similar study found that SA inhibits AHL production by Rh1I in *P. aeruginosa* and that two other common plant phenolics, trans-cinnamaldehyde and tannic acid have the same inhibitory effect in *P. aeruginosa* (Chang et al. 2014)

Carvacrol, monoterpenoid phenol inhibits *Chromobacterium violaceum cviI* gene expression. *cvil* gene encodes for the AHL synthase, signifying that this carvacrol obstructs the production of AHL molecules (Tapia-Rodriguez et al., 2017). Similar effects were found against plant pathogens including *Pectobacterium caratovorum* subspp. *brasiliense* (*Pcb*) and *Pectobacterium aroidearum*, where this compound reduced QS signal molecules productions and inhibits expression QS related genes (Joshi et al. 2016). Moreover, this compound is shown to directly relate with transcriptional regulator (ExpR) and homoserine lactone synthase (ExpI). Docking scores of the compound helps in binding to ExpI/ExpR and, therefore, as a potential QS inhibitor compound (Joshi et al. 2016).

Flavonoids, another group of natural compounds wit diverse phenolic structures, found in stem, roots, flowers, roots, bark vegetable and fruits. The flavonoid derivative, chalcones and its isomers compounds exhibit strong inhibition toward the enzymes secreted by *P. aeruginosa* through QS (Kerekes et al. 2013; Kim et al. 2015). Several other related studies have demonstrated that flavonoids particularly inhibit QSthoiugh antagonism of the autoinducers binding receptors, RhlR and LasR. The presence of flavone A-ring back bone play a key role in potent inhibition of LasR/RhiR DNA binding in *P. aeruginosa*, *C. violaceum, Escherichia coli* and *Staphylococcus aureus* (Liu et al. 2017; Manner and Fallarero 2018; Górniak et al. 2019; Cushnie and Lamb 2011; Paczkowski et al. 2017).

another group of natural substances with variable phenolic structures, are found in fruits, vegetables, grains, bark, roots, stems, flowers, tea and wine. The flavonoid derivative, chalcones and its isomers compounds exhibit strong inhibition toward the enzymes secreted by *P. aeruginosa* through QS (Kim et al. 2015; Kerekes et al. 2013). Several other studies have demonstrated that flavonoids specifically inhibit quorum sensing via antagonism of the autoinducers binding

receptors, LasR, RhlR, the presence of flavone A-ring back bone are essential for potent inhibition of LasR/RhiR DNA binding in *P. aeruginosa*, *C. violaceum, Escherichia coli* and *Staphylococcus aureus* (Liu et al. 2017; Paczkowski et al. 2017; Manner and Fallarero 2018; Górniak et al. 2019; Cushnie and Lamb 2011).

Fenugreek (*Trigonella foenum-graecum* L.) belongs to family Fabaceae is well known for its medicinal properties. Seed extract of fenugreek was tested and revealed that methanolic fraction of the extract inhibits AHL by attenuating virulence such as, protease, LasB elastase, chitinase, extracellular polymeric substances (EPSs) and swarming motility of *P. aeruginosa* PAO1 (Husain et al. 2015). Garlic, one of the widely accepted herbs globally also contains many antimicrobial metabolites. Crushed garlic contains ajoene and several other organosulfide compounds. Ajoene has shown effective antimicrobial activities towards many Gram-negative and Gram-positive bacteria including *Xanthomonas* spp, *Klebsiella pneumoniae* and *E. coli* (Naganawa et al. 1996). Ajoene also attenuates the virulence related genes of *P. aeruginosa* by reducing the expression of important QS related virulence genes mediated through LasR and RhIR (Jakobsen et al. 2012).

Indole alkaloids are one of the major sub-class of alkaloids found in nature that contain structural moiety of indoles, many of them also include isoprene group and are thus called terpene indoles. Indole-3- carbinol, an indole alkaloid commonly found in cruciferous vegetables, reduce virulence of *P. aeruginosa* by lowering the expression of QS related genes and inhibit biofilm formation in *E. coli* (Lee et al. 2011). Another major class of alkaloids is steroidal alkaloids, biosynthesized by the inclusion of one or two nitrogen atom into a steroid molecule. Tomatidine, a steroidal alkaloid blocks the expression of many virulence genes usually induced by QS related genes (*geh*, *muc*, *hla*, *hld*, *plc* and *agr*). This way these compounds interefers with virulence of *S. aureus*, for example hemolysis production (Husain et al. 2015). Erucin and Sulforaphane are

natural isothiocyanates, usually found in cruciferous vegetables such as broccoli. Both compounds inhibit QS activity in *E. coli* and *P. aeruginosa* where they effectively bind with LasR receptor, resulting in inhibition of QS activation genes (Ganin et al. 2013). *Carum copticum* L. is a well-known herb with many pharmacological effects. The essential oil extract of this herb composed of γ-terpinene, thymol, β-pinene and p-cymene has shown significant anti-QS activity against C. *violaceum* (Deryabin et al. 2019).

Sesquiterpenes are the main constituent of essential oils (e.g., citrus fruits, spices and herbs) and have many ecological functions in plants, including as allelopathic agents and as repellents herbivores or resistance to plant pathogens (Dudareva et al. 2004; Paré and Tumlinson 1999). Sesquiterpene lactones, a class of sesquiterpenoids that contain lactone ring was reported to reduce the concentration AHL molecules in *P. aeruginosa* ATCC 27853, indicating it as a good candidate for development of antimicrobial agents (Amaya et al. 2012).

Generally, QS inhibiting chemicals can inhibit QS in diverse bacterial species. For example, ginger (*Zingiber officinales*) rhizomes produce many phenolic acids, including 6-gingirol, 6-shoagol and zingerone, all of which inhibit QS activity in *C. violaceum* bioassays (Kumar et al. 2014). 6-gingerol, a pungent oil from ginger has shown to notably reduce biofilm formation and other major virulence factors by binding with QS receptors in *P. aeruginosa* (Kim et al. 2015). This finding led to the investigation of the role of zingerone in AHL productions using different pathogens such as, *Agrobacterium tumefaciens*, *E. coli*, and *P. aeruginosa*. Interestingly, zingerone showed anti-QS activity against all three pathogens as it interferes with ligand receptor activity interactions with QS receptors (PqsR, LasR, RhlR and TraR), hence, proposing a suitable anti-virulent chemical against *P. aeruginosa* infection (Kumar et al. 2015).

3.2. Anti-biofilm activity of plant derived molecules

Biofilms are thick aggregates of microorganisms attached to a substratum embedded within self-produced polysaccharides and cells in a biofilm function in cooperative manner to benefit community (McDougald et al. 2012). Biofilm formation is tightly linked with QS in several pathogens (Barnard et al. 2007; Liu et al. 2008), There are many examples of plant derived molecules that inhibit/ reduce biofilm formation. For example, both eugenol and carvacrol reduce biofilm formation of *Pectobacterium brasiliense and Pectobacterium aroidearum* and it appears to be due to reduction of AHL synthase and QS regulator expression (Joshi et al. 2016).

Gallic acid is a well-known natural antioxidant that reduces biofilm mass in Gram-negative bacteria and Gram-positive bacteria to a lesser extent. This phenolic acid controls biofilm production and inhibits motility of four human pathogenic bacteria including *Listeria monocytogenes, Staphylococcus, E. coli* and *P. aeruginosa* (Borges et al. 2012; Dusane et al. 2015). Salicylic acid was tested against *E. coli* and *S. aureus* and found to control the growth of both bacteria in planktonic and biofilm states (Monte et al. 2014) and since neither of these pathogens encode AHL synthase, the mechanism of inhibition must differ than that reported in *Pectobacterium* spp. (Joshi et al. 2020). Plants produce small molecular hormones for cellular signal transduction in response to development and environmental cues. For example, plant auxin, 3-indoleacetonitrile (IAN) is involved in developmental processes and stress tolerance (Cohen et al. 2003). DNA microarray and whole transcriptomics data analysis showed that plant auxin, IAN inhibits biofilm formation in *E. coli* and reduces virulence in *P. aeruginosa* by down regulating QS related genes (Lee et al. 2011).

Interestingly, fatty acids serve as signaling molecules to inhibit biofilm formation. For example, cis-2- decenoic acid inhibits biofilm formed by several bacterial pathogens, including *S.*

aureus, *E. coli, P. aeruginosa*, *Klebsiella pneumoniae*, *Bacillus subtilis*, *Proteus mirabilis*, and *Streptococcus pyogenes* (Jennings et al. 2012; Sepehr et al. 2014; Marques et al. 2015; Rahmani-Badi et al. 2014). To date, a variety of fatty acids are known to participate in QS related virulence control, such as cis-11-methyl-2-dodecenoic acid, trans-2-decenoic acid, cis-2-dodecenoic acid, cis-10-methyl-2-dodecenoic acid, cis-11-methyldodeca-2,5-dienoic acid (Cui et al. 2019; He et al. 2010; Beaulieu et al. 2013; Ionescu et al. 2016; Huang and Lee Wong 2007; Ling et al. 2019). Exogenous application of oleic acid (cis-9-octadecenoic acid) inhibits bacterial adhesion and hence biofilm formation of many *S. aureus* strains, and this must occur through a non-AHL-based mechanism in this Gram-positive species (Rabin et al. 2015; Stenz et al. 2008). Further investigation of oleic acid showed that it completely inhibits bacterial biofilm formation of *P. aeruginosa*, specifically by interfering with LuxR, which serves as transcriptional activator protein and blocks AHLcontrolled QS (Singh et al. 2013). Recently an omega fatty acid, petroselinic acid (cis-6-octadecenoic acid) has been discovered to prevent QS regulated virulence, protease and biofilm production by downregulating genes including QS regulator gene (*bsmB),* flagellar transcription regulatory genes *(flhD, fimC* and *fima*) which encode for fimbriae production (Ramanathan et al. 2018).

Oleic acid is the most abundant among all the natural fatty acids and also present in all lipids. It is the principal fatty acid found in ripe fruit (*Olea europaea*) of olive oil . Oleic acid significantly inhibits biofilm development by inhibiting the number of cells of *Staphylococcus aureus* and inhibit biofilm accumulation in *Streptococcus mutans* (Stenz et al. 2008; Pandit et al. 2015). Linoleic acid, which is structurally related with oleic acid also reduces biofilm formation in *Streptococcus mutans* and in *K. pneumoniae* (Jung et al. 2014; Magesh et al. 2013). It strongly diminishes dry weight, EPS production and thickness of *Streptococcus* mutants' biofilm (Jung et

al. 2014). Unsaturated fatty acids, including palmitoleic and myristoleic acid, also inhibit the expression of QS transcription regulators by reducing autoinducers synthesis and therefore biofilm formation of *Acinetobacter baumannii* (Nicol 2018). *Ginkgo biloba* is among the oldest living species of tree and has been used to cure dementia and other circulatory disorders. The phenolic acid from *Ginkgo biloba* has shown significantly reduce biofilm formation in *E. coli* and *S. aureus* without interfering with bacterial growth (Lee et al. 2014). Other phytochemicals including 1,3,4-oxadiazolen, 7-Hydroxyindole and solenopsin A has shown to reduce quorum sensing and hence reduce biofilm formation by interfering with transcriptional regulators of QS (*pqsR* and *rhI)* and *Pseudomonas* quinolone signal (PQS) system (Zender et al. 2013; Lee et al. 2009; Park et al. 2008).

Other plant derived indoles, such as indole-3-carboxyaldehyde, indole-3-acetamide and 3-Indolylacetonitrile significantly reduce the ability of *P. aeruginosa* to form biofilm (Lee et al. 2012). 3-indolylacetonitrile isolated from cruciferous vegetables was further investigated and reported as a potential inhibitor of biofilm production in both *E. coli* and *P. aeruginosa* by reducing EPS production and reduction of curli formation (Lee et al. 2011). Aporphinoid alkaloids including oliverine, iriodenine and pachypodanthine inhibit the biofilm formation of *Yersinia enterocolitica,* a foodborne human pathogen, without reducing the growth of the bacteria. Moreover, pachypodanthine was further tested to reduce QS by inhibiting AHL production in the extracellular cell and hence inhibit biofilm formation in *Y. enterocolitica* (Marco et al. 2020).

3.3. Role of plant derived molecules on motility

Many phytopathogenic bacteria use flagellar motility during infection and this motility contributes to virulence (Jahn et al. 2008; Chesnokova et al. 1997; Mulholland et al. 1993). Several animal and plant pathogens, including *Salmonella*, *Pectobacterium* spp. and *E. coli,* are motile during host-pathogen interactions. Conversely, plant derived compounds have been shown to

interfere with bacterial motility and, therefore, reduce pathogenicity. In some cases, motility inhibition may occur through QS inhibitions. For example, coumarin inhibits swarming motility of *P. aeruginosa* by reducing QS genes related genes *rhII* and *pqsA*.

Transcriptional profile of *E. coli* treated with coumarins showed that when applied at 50 μg/ml repressed curli genes, motility genes, fimbriae production, swarming motility and hence biofilm formations in *E. coli* O157:H7 (Lee et al. 2014). Piperine is an alkaloid found in *Piper nigrum* and reserpine extracted from dried roots of *Rauwoflia serpentine* were tested against *E. coli's* CFT073 ability to colonize under abiotic conditions. Both compounds under sub-inhibitory concentration significantly reduced swimming and swarming motility by inhibiting the expression motility genes (*fimA, papA* and *uvrY*) flagellar gene such as *fliC* (Dusane et al. 2014). They were also reported to reduce swarming and swimming ability of *E. coli* but did not reduce sliding motility of *S. aureus* (Monte et al. 2014). Caffeine (1,3,7-trimethylxanthine) is among few plant products that the general public is very familiar. Caffeine inhibits swarming motility of *P. aeruginosa* by limiting the bacterial colonies with undefined and short tendrils (Husain et al. 2015; Norizan et al. 2013). This inhibition in motility may be due to anti-QS properties of caffeine (Husain et al. 2015). Whole-transcriptomic data of *P. aeruginosa* showed that 3-indolylacetonitrile reduce genes tightly linked with virulence (*pqsE* and *pvcC*) and genes required for motility (*z2200*, *motD, flhF*, and *pilI*) in *P. aeruginosa* and therefore reduce virulence (Lee et al. 2011). IAN also represses motility, virulence related genes and other small molecules transporters in *P. aeruginosa* (Lee et al. 2011).

Fatty acids also inhibit bacterial motility. For instance, 11-methyldodecanoic partly inhibits *P. aeruginosa* swarming motility, while vaccenic and oleic acid are known to completely inhibit motility without reducing bacterial growth (Inoue et al. 2008). Further investigation was conducted

to determine the inhibition mechanisms fatty acids. Myristic acid, lauric acid, stearic acid and palmitic acid were tested against *Proteus mirabilis* virulence related genes. All compounds inhibit swarming motility of *P. mirabili*, however lauric acid, myristic acid, and palmitic acid were not able to inhibit swarming motility of *rsb*A defective mutant (Liaw et al. 2004). *rsb*A regulates swarming behavior which encodes for bacterial two-component signaling system. On the other hand, stearic acid reduces swarming motility in *rsbA* defective mutant, indicating it might occur through Rsb-A independent pathway. Therefore, fatty acids serves as intracellular signals to mimic bacteria and control swarming motility and hence control the expression of virulence factors through either RsbA depend or independent pathways (Liaw et al. 2004). In other cases, the mechanism of motility inhibition is unclear. For example, gallic acid, under subinhibitory concentrations, interrupts three different types of motilities such as, swimming, swarming and twitching of *P. aeruginosa* and swarming and swimming motilities in *E. coli* (Borges et al. 2014; O'May and Tufenkji 2011)

3.4. Plant cell wall degrading enzymes, effector proteins and plant secretion system

To cause disease successfully, many plant pathogens are dependent on production of extracellular enzymes that are capable of degrading plant tissue. Such as, soft rot bacteria including genus *Dickeya* and *Pectobacterium* relies on QS for PCWDEs regulations. Many plants derived phenolic acids act as inhibitors of exoenzymes production in *Pectobacterium* (Joshi et al. 2016; Joshi et al.2015; Joshi et al. 2016). Salicylic acid, tannin and catechin are reported to reduce production of elastase and protease of *Pseudomonas* (Prithiviraj et al., 2005; Vandeputte et al., 2010). Many nightshade family members including potatoes and *Datura stramonium L.* consist of steroidal alkaloids, such as α-Solamarine, α-Solanine, β-Chaconine, Saponin and tropane alkaloids including Calystegine A3/A6/A7 and Calystegine B2/B5 (Joshi et al. 2020; Christhudas

et al. 2012). Stem and tuber extract of M6, wild diploid potato species of *Solanum chacoense* composed of these steroidal and tropane alkaloids. These compounds significantly inhibit QS activity by reducing AHL synthesis and expression of *pel1*, *pel2*, *prt1* and *perE* genes of *Pcb* (Joshi et al. 2020).

Monoterpenes such as thymol and its isomer carvacrol have been reported to strongly inhibit enzymatic secretion such as lipase and coagulase production in *S. aureus* (Souza et al. 2013). Reduction in enzymatic activity may result either from the direct interaction of compounds or it may prevent protein secretion (Souza et al. 2013). The hydroxyl groups in terpenoids such as carvacrol, thymol, terpineol and eugenol are extremely reactive and develop hydrogen bond with different active sites of enzymes and therefore deactivate them (Ouattara et al. 1997; Kim et al. 1995).

Bacterial plant pathogens encode protein secretion systems dedicated to secretion of virulence proteins into the plant apoplast or directly into plant cells (Green and Mecsas 2016). In several cases, expression of these secretion systems or the proteins secreted through them are controlled by QS (Asfour 2018; Hneke and Bassler 2004; Ruwandeepika et al. 2015). For example, QS controls expression of PCWDEs, such as the metalloproteases secreted through the type I secretion system (T1SS) and the pectinases secreted through the type II secretion system (T2SS) by soft rotting *Pectobacteriaceae* plant pathogens. QS may also regulate proteins secreted through the T3SS, such as DspA/E and helper/harpin proteins (Kim et al. Johnson et al. 2006; 2011; Charkowski et al. 2012). The QS dependent T3SS includes the *hrp* cluster with constituents of the structural apparatus, the helpers HrpW, HrpN and effector DspA/E and some other few regulators *hrpL*, *hrpS* and *hrpY*. This indicate that T3SS can be among the major targets to control plant pathogenic bacteria to attenuate disease. Plant derived inhibiting the expression of T3SS

components and helper genes (*hrpA, hrpS*, *hrpL*, *hrpN*, and *rpoN*) in *Dickeya dadantii* (Asfour 2018; Li et al. 2009; Yamazaki et al. 2012; Li et al. 2015).

AraC is a global transcription regulator which controls the expression of many virulence-associated genes of pathogenic bacteria (Yang et al. 2011). Cis-9-octadecenoic acid and cis-9-hexadecenoic acid inhibit VirF in *Y. enterocolitica*, HilD in *S. enterica* and Rns in *E. coli*, whay are AraC like regulatory proteins (Golubeva et al. 2016). Further investigation on exogenous application of cis-9-octadecenoic acid revealed that it inactivates the expression of HilD, a transcription regulator and β-oxidation pathway, therefore reducing the expression of T3SS in *Salmonella* (Golubeva et al. 2016; Boyen et al. 2008). In addition, oleic acid also inactivates TfmR, another transcriptional regulator, led to down regulating HrpG/HrpX, which is the T3SS master regulator, and hence reduced virulence (Teper et al. 2019).

3.5. Pigments, toxins and other virulence factors

Several Gram-negative bacterial species produce violacein, a natural purple pigment which is one of the QS dependent phenotypes (McClean et al. 1997; Gopu et al. 2015). Conversely, plant derived terpenoids including, α-terpineol and cis-3-nonen-1-ol have been shown to exhibit >90% violacein inhibition, suggesting these compounds can be used as a potential QS inhibitor against *C. violaceum* and *P. aeruginosa* (Ahmad et al. 2015). Carvacrol, monoterpene also inhibits violacein production in *C. violaceum* (Burt et al. 2014). Further research on carvacrol has explored that in addition to inhibiting QS-dependent violacein biosynthesis, it regulates QS-controlled pyocyanin production and chitinase activity in *P. aeruginosa* (Burt et al. 2014; Tapia-Rodriguez et al. 2017).

Essential oils can be categorized into two groups based on their inhibitory effects on microbes, slow acting compounds and fast acting compounds. Carvacrol, geraniol, linalool, and

terpinen-4-ol have been categorized as fast-acting compounds. These compounds kill *E. coli* almost on direct contact with bacteria (Friedman et al. 2004). In addition, geraniol, terpineol, citronellol and eugenol inactivate *E. coli* in 2 hours period and hence also categorize as fast acting compounds (Guimarães et al. 2019). Seven wine terpenoids including α-pinene, limonene, myrcene, geraniol, linalool, nerol, and terpineol exhibit high antibacterial activities under minimum inhibitory concentration as these compounds are highly toxic to the pathogen and results in killing three foodborne pathogens including *Salmonella enterica, S. aureu*s and *E. coli* (Wang et al. 2019).

Interestingly, Gram-positive bacteria are slightly more sensitive to some plant derived compounds such as terpenoids compared to Gram-negative bacteria, due to hydrophilic cell wall structure of Gram-positive bacteria (Silhavy et al. 2010). Conversely, Gram-negative bacterial cell wall consists of lipo-polysaccharides, which helps in blocking the penetration of hydrophobic component of terpenoids (Beveridge 1999). Numerous plants derived antimicrobial compounds act on bacterial cytoplasmic membrane, which serves as permeability barrier for most plant derived small molecules. In contrast, terpenes use diverse mode of action to defeat bacteria, for example terpenes act on cell membrane of bacteria and induce leakage of K^+ from bacterial cells (Cox et al. 2000). These ions lead to intracellular acidification, which alters bacterial membrane and causes severe cell membrane damage, hence results in cell death (Perumal et al. 2017). For instant, K^+ damages the cell membrane of bacterial pathogen such as *E. coli* and *S. aureus* (Hada et al. 200; Carson et al. 2002; Cox et al. 2001). Other terpenoids exhibit same mechanism such as terpenes alcohol including farnesol, nerolidol, plaunotol have shown anti-bacterial activity against *S. aureus* by damaging cell membrane because of K^+ ions leakage. Therefore, it is hypothesized that the

antibacterial activity of terpenoids is tightly linked with the affinity of lipid layers in the cell membrane.

Saponin, a triterpene glycoside usually found in beans, spinach, quinoa, and other crops including potatoes and sorghum. Saponin extracts from sorghum and potatoes have anti-bacterial activity against pathogenic bacteria including *E. coli* and *S. aureus and P. brasiliense* (Aoki et al. 2010; Joshi et al. 2020).

4. Conclusions and future prospective

With increasing demand of food and the emergence of several new bacterial plant diseases in recent decades, the development of new vegetable varieties with comprehensive resistance to various bacterial pathogens is urgently needed. Exploring plant specialized metabolites has attained a prominent place in plant biology research due to its novel potential to attenuate plant diseases caused by various pathogens. Advances in genomic and molecular tools can allow plant breeders to select for specific genes of interest and traits to develop new varieties. Omics tools such as metagenomics, transcriptomics, proteomics and metabolomics have huge potential of exploring plant defense mechanisms by exploiting the host-microbe interactions and hence incorporating this information in plant breeding programs. Further integration of metabolomic knowledge in plant breeding programs has immense potential in the development of new elite cultivars which will be resistant to diseases. In addition, the combination of metabolomics with other omics tools can help us to deploy genetic attributes of host-microbe interactions to mitigate yield losses caused to pathogens. Future application of metabolomics may include identification of metabolic markers to understand plant metabolic response to pathogens, which will assist in predictions including, approaches like metabolomics-assisted breeding for crop improvement programs, development of high yielding crops, stress tolerant germlines and to create climate smart

crop varieties. Speed breeding is yet another fascinating area where metabolomics is ready to do wonders in development of elite crop cultivars to attain maximum production.

Nevertheless, I believe that further investigated must be done in order to unfold some of the important scientific question associated with small molecules such as:

1. Do metabolites inhibit virulence factors in a species specific manner?
2. Metabolites are known to interfere with QS signaling molecules as discussed above, can plant pathogenic bacteria escape the impact of small molecules by controlling different QS routes?
3. Based on current understanding of plant derived small molecules in response to pathogen and other abiotic stresses, how we can exploit the knowledge to develop new tools for breeding program to eradicate plant pathogenic bacteria?

The real world is fully composed of microbial interactions, it is imperative to further explore the effects of metabolites in plant defense responses. Increasing research about plant-pathogen interactions derived molecules could pave a unique way of designing new tools in our fight against phytopathogens. My metabolomic work with potato and the important pathogen *Dickeya dianthicola*, will aid in answering some of these important questions and should lead to improved management of soft rot and blackleg diseases.

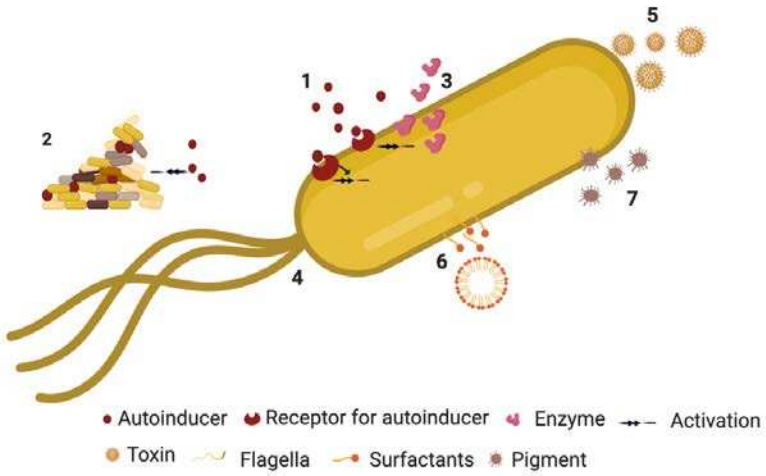
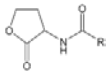
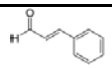
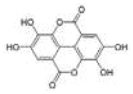
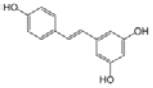
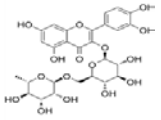
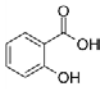
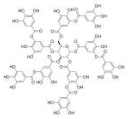
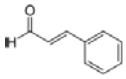
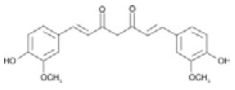
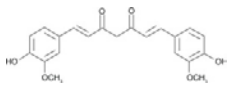
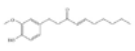
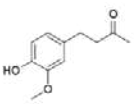
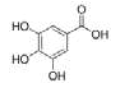
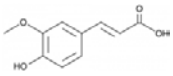
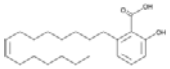
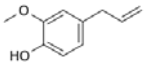
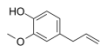
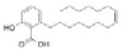
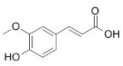
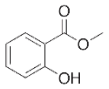
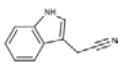
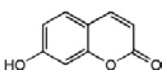
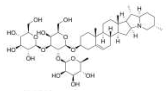
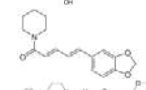
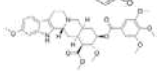
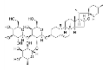
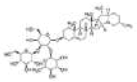
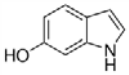
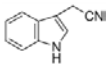


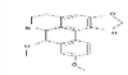
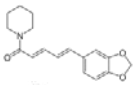
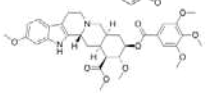
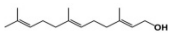
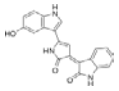
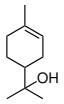
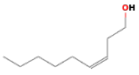
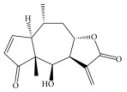
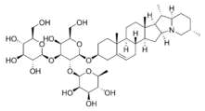
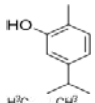
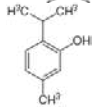
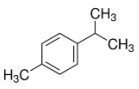
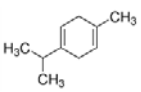
Figure 1.1. Bacterial virulence factors addressed in this review as targets for anti-virulence agents. The plant derived small molecules are active against well recognized pathogenicity factors, such as 1, quorum sensing which regulates other virulence factors, such as 2, bacterial biofilm formation, 3, production of secreted enzymes, and in some cases 4, motility, 5, toxins, 6, surfactant and 7, pigments.

Table 1.1. Summary of plant derived compounds displaying anti-virulence activity

Serial number	Compound	Structure
	Quorum sensing signal	
0	Acyl homoserine lactone	
	Phenolic acids	
1	Cinnamaldehyde	
2	Ellagic acid	
3	Resveratol	
4	Rutin	
5	Salicylic acid	
6	Tannic acid	
7	Trans-cinnamaldehyde	
8	Curcumin	
9	6-gingerol	
10	6-shoagoal	
11	Zingerone	

12	Gallic acid	
13	Ferulic acids	
14	Methylate gallate	
15	Ginkgolic acid	
16	Eugenol	
17	Carvacrol	
18	Eugenol	
19	Ginkgolic acid	
20	Methyl gallate	
21	Ferulic acid	
22	Methyl salicylate	
23	Salicylamide	
	Alkaloids	
1	3-Indoleacetonitrile	
2	7-hydroxycoumarins	
3	Indole-3-carbinol	

4	Saponin	
5	Piperine	
6	Reserpine	
7	Caffeine	
8	α-Solamarine	
9	α-Solanine	
10	b-Chaconine	
11	1,3,4-oxadiazolen	
12	Solenopsin A	
13	Tomatidine	
14	Sulforaphane	
15	Erucin	
16	3-indoleacetonitrile	
17	Indole-3-carboxyaldehyde	
18	Indole-3-acetamide	
29	Pachypodanthine	
20	Iriodenine	

21	Oliverine	
22	Piperine	
23	Reserpine	
2	3-indolylacetonitrile	
	Terpenoids	
1	Farnesol	
2	Violacein	
3	α-Terpineol	
4	Cis-3-nonen-1-ol	
5	Sesquiterpene lactones	
6	Saponin	
7	Carvacrol	
8	Thymol	
9	p-cymene	
10	γ-terpinene	

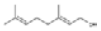
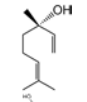
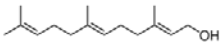
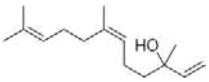
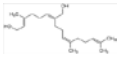
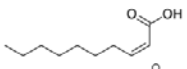
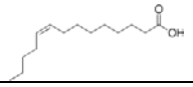
11	β-pinene	
12	geraniol	
13	linalool	
14	Eugenol	
15	Farnesol	
16	Nerolidol	
17	Plaunotol	
	Fatty acids	
1	Oleic acid	
2	Linoleic acid	
3	Dodecanoic acid	
4	lauric acid	
5	Myristic acid	
6	Palmitic acid	
7	Stearic acid	
8	cis-2-Decenoic acid	
9	Myristoleic acids	

Table 1.2. Phenolic compounds and their activity against phytopathogens

Compound	Source	Pathogen	Mode of Action	Reference
Glycosylated flavanones	oranges	*Y. enterocolitica*		Truchado et al. 2012
Cinnamaldehyde, ellagic acid		*Y. enterocolitica and Pectobacterium carotovorum*		Truchado et al. 2012
pomegranate extract, resveratrol rutin				
Trans-cinnamaldehyde		*P. aeruginosa*	pyocyanin	Chang et al. 2014
Salicylic acid, tannic acid and trans-cinnamaldehyde		*P. aeruginosa*	RhlI	Chang et al. 2014
curcumin		*P. aeruginosa*		Rudrappa and Bais 2008
6-gingirol, 6-shoagol and zingerone	ginger	*C. violaceum*, *P. aeruginosa*		Kumar et al. 2014
Zingerone	ginger	*A. tumefacien*s, *E. coli* *P. aeruginosa*	TraR LasR PqsR, RhlR	Kumar et al. 2015
6-gingerol	ginger	*P. aeruginosa*	Binds AHL receptor TraR	Kim et al. 2015
salicylic acid		*Pectobacterium*	AHL	Joshi et al. 2020

methyl salicylate and salicylamide	*P. aeruginosa*	protease activity	Amalaradjou et al. 2010; Kumar et al. 2013; Hu et al. 2013
Carvacrol and eugenol		*expI and expr,* QS regulators	Joshi et al. 2016
Gallic acid	*E. coli*, *P. aeruginosa*, *L. monocytogenes* and *Staphylococcus* spp.	QS regulatory genes	Borges et al. 2012; Dusane et al. 2015
coumarin	*P. aeruginosa*	*rhII* and *pqsA*	Borges et al. 2014; O'May and Tufenkji 2011

Table 1.3. Alkaloids and their role as phytopathogens

Chemicals	Plant sources	Target microorganism	Genes effected	Citation
Indole-3- carbinol	cruciferous vegetables	*E. coli, P. aeruginosa*	QS related genes	Lee et al. 2011
Tomatidine	Solanaceous vegetables	*S. aureus*	QS accessory gene	Husain et al. 2015
Sulforaphane and erucin	broccoli	*P. aeruginosa* and *E. coli*	QS activity	Ganin et al. 2013
3-indolylacetonitrile		*E. coli* and *P. aeruginosa*	EPSs production	Lee et al. 2011
oliverine, iriodenine and pachypodanthine		*Y. enterocolitica*	AHLs concentration	Marco et al. 2020
Fenugreek extract	Fenugreek	*P. aeruginosa*	inhibits AHL, swarming motility	Husain et al. 2015
Ajoene extract		*P. aeruginosa*	LasR and RhIR	Jakobsen et al. 2012
Piperine and reserpine	*Piper nigrum and Rauwoflia serpentine*	*E. coli*	flagellar gene (*fliC*) and motility genes (*fimA, papA* and *uvrY*)	Dusane et al. 2014
1,3,7-trimethylxanthine	Caffeine	*P. aeruginosa*	QS properties	Norizan et al. 2013; Husain et al. 2015

Calystegine A3/A6/A7 and Calystegine B2/B5	Potato tubes	*Pectobacterium* spp.	AHL synthesis, *pel1*, *pel2*, *prt1* and *perE* genes	Joshi et al. 2020
α-Solamarine, α-Solanine, β-Chaconine, Saponin	Potato tuber and *Datura stramonium L*	*Pectobacterium* spp.	AHL synthesis	Joshi et al. 2020; Christhudas et al. 2012
1,3,4-oxadiazolen, 7-Hydroxyindole and solenopsin A		*Pseudomonas spp.*	QS regulatory genes (pqsR and *rhI)*	Zender et al. 2013; Lee et al. 2009; Park et al. 2008
3-indoleacetonitrile		*E. coli, P. aeruginosa*	QS related genes	Lee et al. 2011

Table 1.4. Role of terpenes extracted from essential oil extract against pathogen adapted from (Mehnaz et al. 2019)

Plant species	Common names	Pathogens Tested	Citation
Eugenia caryophyllata	Clove	*Burkholderia cepacia* complex	Maida et al. 2014
Origanum vulgare	Oregano	*B. cepacia* complex	Maida et al. 2014
Thymus vulgaris	Thyme	*B. cepacia* complex	Maida et al. 2014
Mentha spicata	Spearmint	*E. coli*	Shrigod et al. 2017
Mentha spicata and Cymbopogon citratus	Spearmint Lemongrass	*S. aureus* *Acinetobacter baumannii*	Adukwu et al. 2016
Foeniculum vulgare		*B. cepacia* complex	Vasireddy et al. 2018
		Prototheca zopfii	Grzesiak et al. 2016
Eugenia caryophyllata	Clove	*S. typhimurium*	Rafiq et al. 2016
Pelargonium graveolens	Geranium	*E. coli* *Campylobacter* spp.	Kurekci et al. 2013
Laurus nobilis	Bay laurel	*Campylobacter* spp. *Campylobacter* spp.	Kurekci et al. 2013
		S. aureus	de Rapper et al. 2016
Backhousia citriodora Lavandula angustifolia	Lemon myrtle Lavender	*Pseudomonas* spp.	Garzoli et al. 2018

Backhousia citriodora	Lemon myrtle	*Pseudomonas spp.* *E. coli*	Kačániová et al. 2017
Lavandula angustifolia	Lavender		
		Pseudomonas spp.	Zrira and Ghanmi 2016

Table 1.5. Effects of fatty acids as anti-virulence agents against plant pathogens adapted from (Kumar et al. 2020)

Fatty acids	Natural source	*Target miacroorganism*	Growth	Process and genes affected	Citation
Caprylic acid	Milk, palm and kernel oil	*Klebsiella pneumoniae*	Inhibited	Capsule production and cell adhesion	Gupta et al. 2020
Undecylic acid		*Serratia marcescens*	Unchanged	QS-dependent virulence factors	Salini et al. 2015
	Human sweat and breast milk	*C. albicans*, *Candida glabrata*, *Candida tropicalis*, *C. albicans* clinical isolates	Inhibited	Virulence genes	Muthamil et al. 2018
Lauric acid	Coconut oil, laurel oil and palm oil	*Clostridium difficile*	Inhibited	Cell membranes and bacterial adhesins	Yang et al. 2018
Myristic acid	Palm oil, bovine milk and butterfat	*Pseudomonas aeruginosa* PAO1	Unchanged	Unknown	Wenderska et al. 2011
Sarcinic acid		*P. aeruginosa* PAO1	Unchanged	Production of flagella and surface polysaccharides	Inoue et al. 2008
Isopentadecylic acid	Traditional soy fermentate	*P. aeruginosa* PAO1	Unchanged	Unknown	Inoue et al. 2008
Palmitic acid	Palm oil, butter, milk and soybean oil	*Vibrio* spp.	Unchanged	AI-2-based QS	Santhakumari et al. 2017
		P. aeruginosa PAO1	Unchanged	Unknown	Wenderska et al. 2011
		Escherichia coli	Unchanged	Unknown	Wenderska et al. 2011
Montanic acid		*Streptococcus mutans UA159*	Unchanged	Unknown	Khan et al. 2012
cis-2-Decenoic acid		*Staphylococcus aureus*	Inhibited	Unknown	Jennings et al. 2012

		K. pneumoniae	Unchanged	Biofilm dispersal	Rahmani-Badi et al. 2014
Myristoleic acid	*Serenoa repens* extract	*Acinetobacter baumannii*	Unchanged	Unknown	Nicol 2018
Palmitoleic acid	Sea buckthorn oil and macadamia oil	*A. baumannii*	Unchanged	QS genes and material interfaces	Nicol 2018
Petroselinic acid	Parsley seed oil	*S. marcescens*	Unchanged	QS genes	Ramanathan et al. 2018
Linoleic acid	Safflower oil, grapeseed oil and centipede oil	*K. pneumonia*	Inhibited	Unknown	Hobby et al. 2019
α-Linolenic acid	Safflower oil, grapeseed oil and centipede oil	*S. aureus* MSSA 6538	Inhibited	Unknown	Kim et al. 2020
Gondoic acid)	Jojoba oil, plant oil and nuts	*S. aureus*	Unchanged	Hemolytic activity	Lee et al. 2017
cis, cis-11,14-Eicosadienoic acid	Coriander oil and camelina oil	*S. aureus* MSSA 6538	Unchanged	Hemolytic activity	Lee et al. 2017
All-*cis*-5,8,11,14,17-Eicosapentaenoic acid	Herring oil, salmon and algae	*S. aureus*	Unchanged	Expression of α-hemolysin	Kim et al. 2018)
Erucic acid	Herring oil and rapeseed oil	*S. aureus*	Unchanged	Expression of α-hemolysin	Kim et al. 2018
All-*cis*-4,7,10,13,16,19-docosahexaenoic	Herring oil and rapeseed oil	*S. aureus*	Unchanged	Expression of α-hemolysin	Kim et al. 2018

REFERENCES

Adukwu E.C., Bowles M., Edwards-Jones V., Bone H. 2016. Antimicrobial activity, cytotoxicity and chemical analysis of lemongrass essential oil (*Cymbopogon flexuosus*) and pure citral. Appl. Microbiol. Biotechnol. 100:9619–9627.

Ahmad, A., Viljoen, A. M., and Chenia, H. Y. 2015. The impact of plant volatiles on bacterial quorum sensing. Lett. Appl. Microbiol. 60:8–19.

Ahuja, I., Kissen, R., and Bones, A. M. 2012. Phytoalexins in defense against pathogens. Trends Plant Sci. 17:73–90.

Amalaradjou, M. A. R., Narayanan, A., Baskaran, S. A., and Venkitanarayanan, K. 2010. Antibiofilm effect of trans-cinnamaldehyde on uropathogenic *Escherichia coli*. J. Urol. 184:358–363.

Amaya, S., Pereira, J. A., Borkosky, S. A., Valdez, J. C., Bardón, A., and Arena, M. E. 2012. Inhibition of quorum sensing in *Pseudomonas aeruginosa* by sesquiterpene lactones. Phytomedicine. 19:1173–1177.

Aoki, S. K., Diner, E. J., de Roodenbeke, C. t'Kint, Burgess, B. R., Poole, S. J., Braaten, B. A., et al. 2010. A widespread family of polymorphic contact-dependent toxin delivery systems in bacteria. Nature. 468:439.

Arts, I. C. W., and Hollman, P. C. H. 2005. Polyphenols and disease risk in epidemiologic studies. Am. J. Clin. Nutr. 81:317S-325S.

Asfour HZ. 2018. Anti-quorum sensing natural compounds. J Microsc Ultrastruct. 6:1-10

Beaulieu, E. D., Ionescu, M., Chatterjee, S., Yokota, K., Trauner, D., and Lindow, S. 2013. Characterization of a Diffusible Signaling Factor from *Xylella fastidiosa*. mBio. 4. e00539-12

Beveridge, T. J. 1999. Structures of Gram-negative cell walls and their derived membrane vesicles. J. Bacteriol. 181:4725–4733.

Bhattacharya, A., Sood, P., and Citovsky, V. 2010. The roles of plant phenolics in defense and communication during *Agrobacterium* and *Rhizobium* infection. Mol. Plant Pathol. 11:705–719.

Biała, W., and Jasiński, M. 2018. The phenylpropanoid case – it is transport that matters. Front. Plant Sci. https://doi.org/10.3389/fpls.2018.01610

Borges, A., Ferreira, C., Saavedra, M. J., and Simões, M. 2013. Antibacterial activity and mode of action of ferulic and gallic acids against pathogenic bacteria. Microb Drug Resist. 19:256–265.

Borges, A., Saavedra, M. J., and Simões, M. 2012. The activity of ferulic and gallic acids in biofilm prevention and control of pathogenic bacteria. Biofouling. 28:755–767.

Borges, A., Saavedra, M. J., and Simões, M. 2014. Antibiofilm activity of plant molecules against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Planta Med. 80. P1L120.

Borges, A., Serra, S., Cristina Abreu, A., Saavedra, M. J., Salgado, A., and Simões, M. 2014. Evaluation of the effects of selected phytochemicals on quorum sensing inhibition and in vitro cytotoxicity. Biofouling. 30:183–195.

Boue, S. M., Cleveland, T. E., Carter-Wientjes, C., Shih, B. Y., Bhatnagar, D., McLachlan, J. M. et al. 2009. Phytoalexin-enriched functional foods. J. Agric. Food Chem. 57:2614–2622.

Boyen, F., Haesebrouck, F., Vanparys, A., Volf, J., Mahu, M., Van Immerseel, F., et al. 2008. Coated fatty acids alter virulence properties of *Salmonella Typhimurium* and decrease intestinal colonization of pigs. Vet. Microbiol. 132:319–327.

Burt, S. A., Ojo-Fakunle, V. T. A., Woertman, J., and Veldhuizen, E. J. A. 2014. The natural antimicrobial carvacrol inhibits quorum sensing in *Chromobacterium violaceum* and reduces bacterial biofilm formation at sub-lethal concentrations. PLoS One. 9.

Carson, C. F., Hammer, K. A., and Riley, T. V. 2006. *Melaleuca alternifolia* (Tea Tree) Oil: a review of antimicrobial and other medicinal properties. Clin. Microbiol Rev. 19:50–62.

Cebolla-Cornejo, J., Roselló, S., and Nuez, F. 2013. Selection of tomato rich in nutritional terpenes. In natural products: phytochemistry, botany and metabolism of alkaloids, phenolics and terpenes, eds. Kishan Gopal Ramawat and Jean-Michel Mérillon. Berlin, Heidelberg: Springer, p. 2853–2881.

Chang, C.-Y., Krishnan, T., Wang, H., Chen, Y., Yin, W.-F., Chong, Y.-M., et al. 2014. Non-antibiotic quorum sensing inhibitors acting against N -acyl homoserine lactone synthase as druggable target. Sci. Rep. 4:1–8.

Chang, C.-Y., Krishnan, T., Wang, H., Chen, Y., Yin, W.-F., Chong, Y.-M., et al. 2014. Non-antibiotic quorum sensing inhibitors acting against N-acyl homoserine lactone synthase as druggable target. Sci Rep. 4:7245.

Charkowski, A., Blanco, C., Condemine, G., Expert, D., Franza, T., Hayes, C., et al. 2012. The Role of secretion systems and small molecules in soft rot enterobacteriaceae pathogenicity. Annu. Rev. Phytopathol. 50:425–449.

Chen, X., Yu, F., Li, Y., Lou, Z., Toure, S. L., and Wang, H. 2020. The inhibitory activity of p-coumaric acid on quorum sensing and its enhancement effect on meat preservation. CyTA – J. Food. 18:61–67.

Chesnokova, O., Coutinho, J. B., Khan, I. H., Mikhail, M. S., and Kado, C. I. 1997. Characterization of flagella genes of *Agrobacterium tumefaciens*, and the effect of a bald strain on virulence. Mol. Microbiol. 23:579-590.

Christhudas, I. V. S. N., Kumar, P. P., and Agastian, P. 2012. Antimicrobial activity and HPLC analysis of tropane alkaloids in *Streptomyces* spp. isolated from *Datura stramonium* L. Asian J. Pharm. Clin. Res. 5: 278-282.

Cohen, J. D., Slovin, J. P., and Hendrickson, A. M. 2003. Two genetically discrete pathways convert tryptophan to auxin: more redundancy in auxin biosynthesis. Trends Plant Sci. 8:197–199.

Coleman, J. J., White, G. J., Rodriguez-Carres, M., and Vanetten, H. D. 2011. An ABC transporter and a cytochrome P450 of *Nectria haematococca* MPVI are virulence factors on pea and are the major tolerance mechanisms to the phytoalexin pisatin. Mol. Plant Microbe Interact. 24:368–376.

Comino, C., Lanteri, S., Portis, E., Acquadro, A., Romani, A., Hehn, A., et al. 2007. Isolation and functional characterization of a cDNA coding a hydroxycinnamoyl transferase involved in phenylpropanoid biosynthesis in *Cynara cardunculus* L. BMC Plant Biol. 7:14.

Cortinovis, C., and Caloni, F. 2015. Alkaloid-containing plants poisonous to cattle and horses in Europe. Toxins (Basel). 7:5301–5307.

Cox, S. D., Mann, C. M., Markham, J. L., Gustafson, J. E., Warmington, J. R., and Wyllie, S. G. 2001. Determining the antimicrobial actions of tea tree oil. Molecules. 6:87–91.

Cox, S. D., Mann, C. M., Markham, J. L., Gustafson, J. E., Warmington, J. R., and Wyllie, S. G. 2001. Determining the antimicrobial actions of tea tree oil. Molecules. 6:87–91.

Cugini, C., Calfee, M. W., Farrow, J. M., Morales, D. K., Pesci, E. C., and Hogan, D. A. 2007. Farnesol, a common sesquiterpene, inhibits PQS production in *Pseudomonas aeruginosa*. Mol. Microbiol. 65:896–906.

Fuqua C, Parsek MR, Greenberg EP. 2001. Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. Annu Rev Genet. 35:439-68

Fuqua WC, Winans SC, Greenberg EP. 1994. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. J Bacteriol. 176(2):269-75

Cui, C., Song, S., Yang, C., Sun, X., Huang, Y., Li, K., et al. 2019. Disruption of quorum sensing and virulence in *Burkholderia cenocepacia* by a structural analogue of the *cis*-2-dodecenoic acid signal. Appl. Environ. Microbiol. 85.

Cui, C., Song, S., Yang, C., Sun, X., Huang, Y., Li, K., et al. 2019. Disruption of quorum sensing and virulence in *Burkholderia cenocepacia* by a structural analogue of the cis-2-dodecenoic Acid Signal. Appl. Environ. Microbiol. 85.

Cushnie, T. P. T., and Lamb, A. J. 2011. Recent advances in understanding the antibacterial properties of flavonoids. Int. J. Antimicrob. Agents. 38:99–107.

Cushnie, T. P. T., Cushnie, B., and Lamb, A. J. 2014. Alkaloids: An overview of their antibacterial, antibiotic-enhancing and antivirulence activities. Int. J. Antimicrob. Agents 44:377-386.

Davey, M. E., and O'toole, G. A. 2000. Microbial biofilms: from ecology to molecular genetics. Microbiol. Mol. Biol. Rev. 64:847–867.

Davies, D. G., Parsek, M. R., Pearson, J. P., Iglewski, B. H., Costerton, J. W., and Greenberg, E. P. 1998. The involvement of cell-to-Cell signals in the development of a bacterial biofilm. Science. 280:295–298.

De Rapper S., Viljoen A., Van Vuuren S. 2016. The in vitro antimicrobial effects of *Lavandula angustifolia* essential oil in combination with conventional antimicrobial agents. Evidence-Based Complement. Altern. Med. doi: 10.1155/2016/2752739

Deryabin, D., Galadzhieva, A., Kosyan, D., and Duskaev, G. 2019. Plant-derived inhibitors of AHL-mediated quorum sensing in bacteria: modes of action. Int. J. Mol. Sci. 20:5588.

Desbois, A. P., and Smith, V. J. 2010. Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential. Appl. Microbiol. Biotechnol. 85:1629–1642.

Diaz, G. J. 2015. Toxicosis by plant alkaloids in humans and animals in Colombia. Toxins (Basel). 7:5408–5416.

Diggle, S. P., Matthijs, S., Wright, V. J., Fletcher, M. P., Chhabra, S. R., Lamont, I. L., et al. 2007. The *Pseudomonas aeruginosa* 4-quinolone signal molecules HHQ and PQS play multifunctional roles in quorum sensing and iron entrapment. Chem. Biol. 14:87–96.

Ding, X., Yin, B., Qian, L., Zeng, Z., Yang, Z., Li, H., et al. 2011. Screening for novel quorum-sensing inhibitors to interfere with the formation of *Pseudomonas aeruginosa* biofilm. J. Med. Microbiol. 60:1827–1834.

Dixon, R. A., Achnine, L., Kota, P., Liu, C.-J., Reddy, M. S. S., and Wang, L. 2002. The phenylpropanoid pathway and plant defence—a genomics perspective. Mol. Plant Pathol. 3:371–390.

Doehlemann, G., and Hemetsberger, C. 2013. Apoplastic immunity and its suppression by filamentous plant pathogens. New Phytologist. 198:1001–1016.

Donlan, R. M., and Costerton, J. W. 2002. Biofilms: Survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev. 15:167–193.

Dudareva, N., Pichersky, E., and Gershenzon, J. 2004. Biochemistry of plant volatiles. Plant Physiol. 135:1893–1902.

Dusane, D. H., Hosseinidoust, Z., Asadishad, B., and Tufenkji, N. 2014. Alkaloids modulate motility, biofilm formation and antibiotic susceptibility of pathogenic *Escherichia coli*. PLoS One. 9.

Dusane, D. H., O'May, C., and Tufenkji, N. 2015. Effect of tannic and gallic acids alone or in combination with carbenicillin or tetracycline on *Chromobacterium violaceum* CV026 growth, motility, and biofilm formation. Can. J. Microbiol. 61:487–494.

Fahn, A. 1988. Secretory tissues in vascular plants. New Phytologist. 108:229–257.

Franceschi, V. R., Krokene, P., Christiansen, E., and Krekling, T. 2005. Anatomical and chemical defenses of conifer bark against bark beetles and other pests. New Phytologist. 167:353–376.

Friedman, M., Henika, P. R., Levin, C. E., and Mandrell, R. E. 2004. Antibacterial activities of plant essential oils and their components against *Escherichia coli* O157:H7 and *Salmonella enterica* in Apple Juice. J. Agric. Food Chem. 52:6042–6048.

Ganin, H., Rayo, J., Amara, N., Levy, N., Krief, P., and M. Meijler, M. 2013. Sulforaphane and erucin, natural isothiocyanates from broccoli, inhibit bacterial quorum sensing. MedChemComm. 4:175–179.

Garzoli S., Božović M., Baldisserotto A., Sabatino M., Cesa S., Pepi F., Vicentini C.B., Manfredini S., Ragno R. 2018. Essential oil extraction, chemical analysis and anti-Candida activity of *Foeniculum vulgare* Miller–new approaches. Nat. Prod. Res. 32:1254-1259.

Glas, J. J., Schimmel, B. C. J., Alba, J. M., Escobar-Bravo, R., Schuurink, R. C., and Kant, M. R. 2012. Plant glandular trichomes as targets for breeding or engineering of resistance to herbivores. Int. J. Mol. Sci. 13:17077–17103.

Golubeva, Y., Ellermeier, J., Chubiz, J., and Slauch, J. 2016. Intestinal long-chain fatty acids act as a direct signal to modulate expression of the *Salmonella* pathogenicity island 1 type III secretion system. mBio. 7.

Gopu, V., Kothandapani, S., and Shetty, P. H. 2015. Quorum quenching activity of Syzygium cumini (L.) Skeels and its anthocyanin malvidin against *Klebsiella pneumoniae*. Microb. Pathog. 79:61–69.

Górniak, I., Bartoszewski, R., and Króliczewski, J. 2019. Comprehensive review of antimicrobial activities of plant flavonoids. Phytochem Rev. 18:241–272.

Graf, B. A., Milbury, P. E., and Blumberg, J. B. 2005. Flavonols, flavones, flavanones, and human health: epidemiological evidence. J. Med. Food. 8:281–290.

Gramazio, P., Prohens, J., Plazas, M., Andújar, I., Herraiz, F. J., Castillo, E., et al. 2014. Location of chlorogenic acid biosynthesis pathway and polyphenol oxidase genes in a new interspecific anchored linkage map of eggplant. BMC Plant Biol. 14:350.

Green ER, Mecsas J. 2016. Bacterial secretion systems - an overview. Microbiol Spectr. 4.

Grzesiak B., Głowacka A., Krukowski H., Lisowski A., Lassa H., Sienkiewicz M. 2016. The in vitro efficacy of essential oils and antifungal drugs against *Prototheca zopfii*. Mycopathologia.181:609–615.

Guimarães, A. C., Meireles, L. M., Lemos, M. F., Guimarães, M. C. C., Endringer, D. C., Fronza, M., et al. 2019. Antibacterial activity of terpenes and terpenoids present in essential oils. Molecules. 24:2471.

Gupta, A., Cheepurupalli, L., Vigneswaran, S., Singh Rathore, S., Suma Mohan, S., and Ramakrishnan, J. 2020. In vitro and in silico investigation of caprylic acid effect on multi drug resistant (MDR) *Klebsiella pneumoniae* biofilm. J. Biomol. Struct. Dyn. 38:616–624.

Gupta, S. C., Patchva, S., and Aggarwal, B. B. 2013. Therapeutic roles of curcumin: lessons learned from clinical trials. AAPS J. 15:195–218.

Gutiérrez-Barranquero, J. A., Reen, F. J., McCarthy, R. R., and O'Gara, F. 2015. Deciphering the role of coumarin as a novel quorum sensing inhibitor suppressing virulence phenotypes in bacterial pathogens. Appl. Microbiol. Biotechnol. 99:3303–3316.

Hada, T., Inoue, Y., Shiraishi, A., and Hamashima, H. 2003. Leakage of K+ ions from *Staphylococcus aureus* in response to tea tree oil. J. Microbiol. Methods. 53:309–312.

Hall-Stoodley, L., Costerton, J. W., and Stoodley, P. 2004. Bacterial biofilms: from the natural environment to infectious diseases. Nat. Rev. Microbiol. 2:95–108.

Hammerschmidt, R. 1999. Pytoalxins: What Have We Learned After 60 Years? Annu. Rev. Phytopathol. 37:285–306.

He, Y.-W., Wu, J., Cha, J.-S., and Zhang, L.-H. 2010. Rice bacterial blight *pathogen Xanthomonas oryzae* pv. *oryzae* produces multiple DSF-family signals in regulation of virulence factor production. BMC Microbiology. 10:187.

He, Y.-W., Wu, J., Cha, J.-S., and Zhang, L.-H. 2010. Rice bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae* produces multiple DSF-family signals in regulation of virulence factor production. BMC Microbiol. 10:187.

Henke JM, Bassler BL. 2004. Quorum sensing regulates type III secretion in *Vibrio harveyi* and *Vibrio parahaemolyticus*. J. Bacteriol. 186:3794-805

Henke, J. M., and Bassler, B. L. 2004. Bacterial social engagements. Treds Cell Biol. 14:648–656.

Herrmann, K. M. 1995. The shikimate pathway as an entry to aromatic secondary metabolism. Plant Physiol. 107:7–12.

Hobby, C. R., Herndon, J. L., Morrow, C. A., Peters, R. E., Symes, S. J. K., and Giles, D. K. 2019. Exogenous fatty acids alter phospholipid composition, membrane permeability, capacity for

biofilm formation, and antimicrobial peptide susceptibility in *Klebsiella pneumoniae*. MicrobiologyOpen. doi: 10.1002/mbo3.635

Holland, K. W., and O'Keefe, S. F. 2010. Recent applications of peanut phytoalexins. Recent Pat Food Nutr Agric. 2:221–232.

Holopainen, J. K. 2004. Multiple functions of inducible plant volatiles. Trends in Plant Science. 9:529–533.

Hu, P., Huang, P., and Chen, M. W. 2013. Curcumin reduces *Streptococcus* mutans biofilm formation by inhibiting sortase A activity. Arch. Oral Biol. 58:1343–1348.

Huang, T.-P., and Lee Wong, A. C. 2007. Extracellular fatty acids facilitate flagella-independent translocation by *Stenotrophomonas maltophilia*. Res. Microbiol. 158:702–711.

Huchelmann, A., Boutry, M., and Hachez, C. 2017. Plant glandular trichomes: natural cell factories of high biotechnological interest. Plant Physiol. 175:6–22.

Husain, F. M., Ahmad, I., Khan, M. S., and Al-Shabib, N. A. 2015. Trigonella foenum-graceum (Seed) extract interferes with quorum sensing regulated traits and biofilm formation in the strains of *Pseudomonas aeruginosa* and *Aeromonas hydrophila*. Evid Based Complement Alternat Med. 2015:879540.

Husain, F. M., Ahmad, I., Khan, Mohammad S., Ahmad, E., Tahseen, Q., Khan, Mohd Shahnawaz, et al. 2015. Sub-MICs of Mentha piperita essential oil and menthol inhibits AHL mediated quorum sensing and biofilm of Gram-negative bacteria. Front Microbiol. 6:420.

Inoue, T., Shingaki, R., and Fukui, K. 2008. Inhibition of swarming motility of Pseudomonas aeruginosa by branched-chain fatty acids. FEMS Microbiol. Lett. 281:81–86.

Ionescu, M., Yokota, K., Antonova, E., Garcia, A., Beaulieu, E., Hayes, T., et al. 2016. Promiscuous diffusible signal factor production and responsiveness of the *Xylella fastidiosa* Rpf System. mBio. 7: e01054-16

Jaafar, S. N. S., Baron, J., Siebenhandl-Ehn, S., Rosenau, T., Böhmdorfer, S., and Grausgruber, H. 2013. Increased anthocyanin content in purple pericarp × blue aleurone wheat crosses. Plant Breed. 132:546–552.

Jahangir, M., Kim, H. K., Choi, Y. H., and Verpoorte, R. 2009. Health-affecting compounds in *Brassicaceae*. Compr. Rev. Food Saf. 8:31–43.

Jahn, C. E., Willis, D. K., and Charkowski, A. O. 2008. The flagellar sigma Factor FliA is required for *Dickeya dadantii* Virulence. MPMI. 21:1431–1442.

Jakobsen, T. H., Gennip, M. van, Phipps, R. K., Shanmugham, M. S., Christensen, L. D., Alhede, M., et al. 2012. Ajoene, a sulfur-rich molecule from garlic, inhibits genes controlled by quorum sensing. AAC. 56:2314–2325.

Jennings, J. A., Courtney, H. S., and Haggard, W. O. 2012. Cis-2-decenoic Acid Inhibits *S. aureus* growth and biofilm in vitro: a pilot study. Clin. Orthop. Relat. Res. 470:2663–2670.

Jennings, J. A., Courtney, H. S., and Haggard, W. O. 2012. Cis-2-decenoic acid inhibits *S. aureus* growth and biofilm in vitro: a pilot study. Clin. Orthop. Relat. Res. 470:2663–2670.

Johnson, T. L., Abendroth, J., Hol, W. G., and Sandkvist, M. 2006. Type II secretion: from structure to function. FEMS Microbiol. Lett. 255:175–186.

Joshi JR, Burdman S, Lipsky A, Yariv S, Yedidia I. 2016. Plant phenolic acids affect the virulence of *Pectobacterium aroidearum* and *P. carotovorum* spp. *brasiliense* via quorum sensing regulation. Mol. Plant Pathol. 17:487-500

Joshi JR, Burdman S, Lipsky A, Yedidia I. 2015. Effects of plant antimicrobial phenolic compounds on virulence of the genus *Pectobacterium*. Res. Microbiol. 166:535-45

Joshi JR, Khazanov N, Senderowitz H, Burdman S, Lipsky A, Yedidia I. 2016. Plant phenolic volatiles inhibit quorum sensing in Pectobacteria and reduce their virulence by potential binding to ExpI and ExpR proteins. Sci Rep. 6:38126.

Joshi, J. R., Yao, L., Charkowski, A. O., and Heuberger, A. L. 2020. Metabolites from wild potato inhibit virulence factors of the soft rot and blackleg pathogen *Pectobacterium brasiliense*. MPMI. 34:100-109

Jung, J.-E., Pandit, S., and Jeon, J.-G. 2014. Identification of linoleic acid, a main component of the n-hexane fraction from *Dryopteris crassirhizoma*, as an anti-*Streptococcus* mutans biofilm agent. Biofouling. 30:789–798.

Kačániová M., Terentjeva M., Vukovic N., Puchalski C., Roychoudhury S., Kunová S., Klūga A., Tokár M., Kluz M., Ivanišová E. 2017. The antioxidant and antimicrobial activity of essential oils against *Pseudomonas* spp. isolated from fish. Saudi Pharm. J. 25:1108–1116.

Kachroo, A., and Kachroo, P. 2009. Fatty acid–derived signals in plant defense. Annu. Rev. Phytopathol. 47:153–176.

Kang, M.-S., Oh, J.-S., Kang, I.-C., Hong, S.-J., and Choi, C.-H. 2008. Inhibitory effect of methyl gallate and gallic acid on oral bacteria. J. Microbiol. 46:744–750.

Kaushik, P., Andújar, I., Vilanova, S., Plazas, M., Gramazio, P., Herraiz, F. J., et al. 2015. Breeding vegetables with increased content in bioactive phenolic acids. Molecules. 20:18464–18481.

Kerekes, E.-B., Deák, É., Takó, M., Tserennadmid, R., Petkovits, T., Vágvölgyi, C., et al. 2013. Anti-biofilm forming and anti-quorum sensing activity of selected essential oils and their main components on food-related micro-organisms. J. Appl. Microbiol. 115:933–942.

Khan, R., Khanam, Z., and Khan, A. U. 2012. Isolation and characterization of n-octacosanoic acid from *Viburnum foetens*: a novel antibiofilm agent against Streptococcus Mutans. Med. Chem. Res. 21:1411–1417.

Kim, H.-S., Ham, S.-Y., Jang, Y., Sun, P.-F., Park, J.-H., Hoon Lee, J., et al. 2019. Linoleic acid, a plant fatty acid, controls membrane biofouling via inhibition of biofilm formation. Fuel. 253:754–761.

Kim, H.-S., Lee, S.-H., Byun, Y., and Park, H.-D. 2015. 6-Gingerol reduces *Pseudomonas aeruginosa* biofilm formation and virulence via quorum sensing inhibition. Sci. Rep. 5:8656.

Kim, J., Marshall, M. R., and Wei, C. 1995. Antibacterial activity of some essential oil components against five foodborne pathogens. J. Agric. Food Chem. 43:2839–2845.

Kim, Y.-G., Lee, J.-H., Kim, S.-I., Baek, K.-H., and Lee, J. 2015. Cinnamon bark oil and its components inhibit biofilm formation and toxin production. Int. J. Food Microbiol. 195:30–39.

Kim, Y.-G., Lee, J.-H., Park, J. G., and Lee, J. 2020. Inhibition of *Candida albicans* and *Staphylococcus aureus* biofilms by centipede oil and linoleic acid. Biofouling. 36:126–137.

Kim, Y.-G., Lee, J.-H., Raorane, C. J., Oh, S. T., Park, J. G., and Lee, J. 2018. Herring oil and omega fatty acids inhibit *Staphylococcus aureus* biofilm formation and virulence. Front. Microbiol. 9:1241.

Kiokias, S., Proestos, C., and Oreopoulou, V. 2020. Phenolic acids of plant origin—A review on their antioxidant activity in vitro (O/W emulsion systems) along with their in vivo health biochemical properties. Foods. 9:534.

Kirby, J., Nishimoto, M., Chow, R. W. N., Baidoo, E. E. K., Wang, G., Martin, J., et al. 2015. Enhancing terpene yield from sugars via novel routes to 1-Deoxy-d-Xylulose 5-Phosphate. Appl. Environ. Microbiol. 81:130–138.

Kumar, L., Chhibber, S., and Harjai, K. 2013. Zingerone inhibit biofilm formation and improve antibiofilm efficacy of ciprofloxacin against *Pseudomonas aeruginosa* PAO1. Fitoterapia. 90:73–78.

Kumar, L., Chhibber, S., Kumar, R., Kumar, M., and Harjai, K. 2015. Zingerone silences quorum sensing and attenuates virulence of *Pseudomonas aeruginosa*. Fitoterapia. 102:84–95.

Kumar, N. V., Murthy, P. S., Manjunatha, J. R., and Bettadaiah, B. K. 2014. Synthesis and quorum sensing inhibitory activity of key phenolic compounds of ginger and their derivatives. Food Chem. 159:451–457.

Kumar, N. V., Murthy, P. S., Manjunatha, J. R., and Bettadaiah, B. K. 2014. Synthesis and quorum sensing inhibitory activity of key phenolic compounds of ginger and their derivatives. Food Chem. 159:451–457.

Kumar, P., Lee, J.-H., Beyenal, H., and Lee, J. 2020. Fatty acids as antibiofilm and antivirulence agents. Trends Microbiol. 28:753–768.

Kurekci C., Padmanabha J., Bishop-Hurley S.L., Hassan E., Al Jassim R.A.M., McSweeney C.S. 2013. Antimicrobial activity of essential oils and five terpenoid compounds against *Campylobacter jejuni* in pure and mixed culture experiments. Int. J. Food Microbiol. 166:450–457.

Lee, J.-H., Cho, M. H., and Lee, J. 2011. 3-Indolylacetonitrile decreases *Escherichia coli* O157:H7 biofilm formation and *Pseudomonas aeruginosa* virulence. Environ. Microbiol. 13:62–73.

Lee, J.-H., Kim, Y.-G., Cho, H. S., Ryu, S. Y., Cho, M. H., and Lee, J. 2014. Coumarins reduce biofilm formation and the virulence of *Escherichia coli* O157:H7. Phytomedicine. 21:1037–1042.

Lee, J.-H., Kim, Y.-G., Cho, M. H., Kim, J.-A., and Lee, J. 2012. 7-fluoroindole as an antivirulence compound against *Pseudomonas aeruginosa*. FEMS Microbiol Lett. 329:3.

Lee, J.-H., Kim, Y.-G., Cho, M. H., Kim, J.-A., and Lee, J. 2012. 7-fluoroindole as an antivirulence compound against *Pseudomonas aeruginosa*. FEMS Microbiol. Lett. 329:36–44.

Lee, J.-H., Kim, Y.-G., Park, J. G., and Lee, J. 2017. Supercritical fluid extracts of Moringa oleifera and their unsaturated fatty acid components inhibit biofilm formation by *Staphylococcus aureus*. Food Control. 80:74–82.

Lee, J.-H., Kim, Y.-G., Ryu, S. Y., Cho, M. H., and Lee, J. 2014. Ginkgolic acids and ginkgo biloba extract inhibit *Escherichia coli* O157:H7 and *Staphylococcus aureus* biofilm formation. Int. J. Food Microbiol. 174:47–55.

Lee, J.-H., Regmi, S. C., Kim, J.-A., Cho, M. H., Yun, H., Lee, C.-S., et al. 2011. Apple Flavonoid Phloretin Inhibits *Escherichia coli* O157:H7 Biofilm formation and ameliorates colon inflammation in rats. Infect. Immun. 79:4819–4827.

Lee, J., Attila, C., Cirillo, S. L. G., Cirillo, J. D., and Wood, T. K. 2009. Indole and 7-hydroxyindole diminish *Pseudomonas aeruginosa* virulence. Microb. Biotechnol. 2:75–90.

Li Y, Hutchins W, Wu X, Liang C, Zhang C, et al. 2015. Derivative of plant phenolic compound inhibits the type III secretion system of *Dickeya dadantii* via HrpX/HrpY two-component signal transduction and Rsm systems. Mol. Plant Pathol. 16(2):150-63

Li Y, Peng Q, Selimi D, Wang Q, Charkowski AO, et al. 2009. The plant phenolic compound p-coumaric acid represses gene expression in the *Dickeya dadantii* type III secretion system. Appl Environ Microbiol. 75(5):1223-8

Li, Y., Peng, Q., Selimi, D., Wang, Q., Charkowski, A. O., Chen, X., et al. 2009. The plant phenolic compound p-coumaric acid represses gene expression in the *Dickeya dadantii* type III secretion system. Appl Environ Microbiol. 75:1223–1228.

Liaw, S.-J., Lai, H.-C., and Wang, W.-B. 2004. Modulation of swarming and virulence by fatty acids through the RsbA protein in *Proteus mirabilis*. Infect. Immun. 72:6836–6845.

Ling, J., Zhu, R., Laborda, P., Jiang, T., Jia, Y., Zhao, Y., et al. 2019. LbDSF, the lysobacter brunescens quorum-sensing system diffusible signaling factor, regulates anti- *Xanthomonas* XSAC biosynthesis, colony morphology, and surface motility. Front. Microbiol. 10:1230.

Liu H, Coulthurst SJ, Pritchard L, Hedley PE, Ravensdale M, et al. 2008. Quorum sensing coordinates brute force and stealth modes of infection in the plant pathogen *Pectobacterium atrosepticum*. PLoS Pathog. 4:e1000093

Liu, H., Coulthurst, S. J., Pritchard, L., Hedley, P. E., Ravensdale, M., Humphris, S., et al. 2008. Quorum sensing coordinates brute force and stealth modes of infection in the plant pathogen *Pectobacterium atrosepticum*. PLoS Pathog. doi: 10.1371/journal.ppat.1000093.

Liu, Z., Pan, Y., Li, X., Jie, J., and Zeng, M. 2017. Chemical composition, antimicrobial and anti-quorum sensing activities of pummelo peel flavonoid extract. Industrial Crops and Products. 109:862–868.

Liu, Z., Pan, Y., Li, X., Jie, J., and Zeng, M. 2017. Chemical composition, antimicrobial and anti-quorum sensing activities of pummelo peel flavonoid extract. Ind Crops Prod. 109:862–868.

Liu, Z., Pan, Y., Li, X., Jie, J., and Zeng, M. 2017. Chemical composition, antimicrobial and anti-quorum sensing activities of pummelo peel flavonoid extract. IND. 109:862–868.

Lyu, S. W., Blum, U., Gerig, T. M., and O'Brien, T. E. 1990. Effects of mixtures of phenolic acids on phosphorus uptake by cucumber seedlings. J. Chem. Ecol. 16:2559–2567.

Magesh, H., Kumar, A., Alam, A., Priyam, null, Sekar, U., Sumantran, V. N., et al. 2013. Identification of natural compounds which inhibit biofilm formation in clinical isolates of *Klebsiella pneumoniae*. Indian J. Exp. Biol. 51:764–772.

Maida I., Lo Nostro A., Pesavento G., Barnabei M., Calonico C., Perrin E., Chiellini C., Fondi M., Mengoni A., Maggini V., et al. 2014. Exploring the anti- *Burkholderia cepacia* complex activity of essential oils: A preliminary analysis. Evid Based Complement Altern Med. 2014:573518.

Mandal, S. M., Chakraborty, D., and Dey, S. 2010. Phenolic acids act as signaling molecules in plant-microbe symbioses. Plant Signal Behav. 5:359–368.

Manner, S., and Fallarero, A. 2018. Screening of natural product derivatives identifies two structurally related flavonoids as potent quorum sensing inhibitors against Gram-negative bacteria. Int. J. Mol. Sci. 19:1346.

Manner, S., and Fallarero, A. 2018. Screening of natural product derivatives identifies two structurally related flavonoids as potent quorum sensing inhibitors against Gram-negative bacteria. Int. J. Mol. Sci. 19:1346.

Marco, N. I. D., Pungitore, C. R., and Lucero-Estrada, C. S. M. 2020. Aporphinoid alkaloids inhibit biofilm formation of *Yersinia enterocolitica* isolated from sausages. J. Appl. Microbiol. 129:1029–1042.

Marques, C. N. H., Davies, D. G., and Sauer, K. 2015. Control of biofilms with the fatty acid signaling molecule cis-2-Decenoic Acid. Pharmaceuticals. 8:816–835.

Martens, D. A. 2002. Relationship between plant phenolic acids released during soil mineralization and aggregate stabilization. Soil Sci Soc Am J. 66:1857–1867.

Matsuura, H. N., and Fett-Neto, A. G. 2013. The major indole alkaloid N, β-D-glucopyranosyl vincosamide from leaves of Psychotria leiocarpa Cham. & Schltdl. is not an antifeedant but shows broad antioxidant activity. Nat. Prod. Res. 27:402–411.

McClean, K. H., Winson, M. K., Fish, L., Taylor, A., Chhabra, S. R., Camara, M., et al. 1997. Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. Microbiol. 143:3703–3711.

McDougald D, Rice SA, Barraud N, Steinberg PD, Kjelleberg S. 2012. Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. Nat Rev Micro. 10:39-50

Mcmillan, M., and Thompson, J. C. 1979. An Outbreak of suspected solanine poisoning in schoolboys examination of criteria of solanine poisoning. QJM. 48:227–243.

Mewalal, R., Rai, D. K., Kainer, D., Chen, F., Külheim, C., Peter, G. F., et al. 2017. Plant-derived terpenes: a feedstock for specialty biofuels. Trends Biotechnol. 35:227–240.

Mitchell, G., Lafrance, M., Boulanger, S., Séguin, D. L., Guay, I., Gattuso, M., et al. 2012. Tomatidine acts in synergy with aminoglycoside antibiotics against multiresistant Staphylococcus aureus and prevents virulence gene expression. J. Antimicrob. Chemother. 67:559–568.

Monte, J., Abreu, A. C., Borges, A., Simões, L. C., and Simões, M. 2014. Antimicrobial activity of selected phytochemicals against *Escherichia coli* and *Staphylococcus aureus* and their biofilms. Pathogens. 3:473–498.

Morrell, P. L., Buckler, E. S., and Ross-Ibarra, J. 2011. Crop genomics: advances and applications. Nat. Rev. Genet. 13:85–96.

Mostafa, I., Abbas, H. A., Ashour, M. L., Yasri, A., El-Shazly, A. M., Wink, M., et al. 2020. Polyphenols from Salix tetrasperma Impair Virulence and Inhibit Quorum Sensing of *Pseudomonas aeruginosa*. Molecules. 25(6): 1341

Mulholland, V., Hinton, J. C. D., Sidebotham, J., Toth, I. K., Hyman, L. J., Perombelon, M. C. M., Reeves, P. J., and Salmond, G. P. C. 1993. A plei- otropic reduced virulence (Rvi-) mutant of

Erwinia carotovora subspecies *atroseptica* is defective in flagella assembly proteins that are conserved in plant and animal bacterial pathogens. Mol. Microbiol. 9:343-356.

Muthamil, S., Balasubramaniam, B., Balamurugan, K., and Pandian, S. K. 2018. Synergistic effect of quinic acid derived from *Syzygium cumini* and undecanoic acid against *Candida* spp. Biofilm and Virulence. Front Microbiol. 9:2835.

Naganawa, R., Iwata, N., Ishikawa, K., Fukuda, H., Fujino, T., and Suzuki, A. 1996. Inhibition of microbial growth by ajoene, a sulfur-containing compound derived from garlic. Appl. Environ. Microbiol. 62:4238–4242.

Ng, T. B., Ye, X. J., Wong, J. H., Fang, E. F., Chan, Y. S., Pan, W., et al. 2011. Glyceollin, a soybean phytoalexin with medicinal properties. Appl. Microbiol. Biotechnol. 90:59–68.

Nicol, M. J. 2018. The kinetics of the dissolution of malachite in acid solutions. Hydrometallurgy. 177:214–217.

Nicol, M., Alexandre, S., Luizet, J.-B., Skogman, M., Jouenne, T., Salcedo, S. P., et al. 2018. Unsaturated fatty acids affect quorum sensing communication system and inhibit motility and biofilm formation of *Acinetobacter baumannii.* Int. J. Mol. Sci. 19:214.

Niggeweg, R., Michael, A. J., and Martin, C. 2004. Engineering plants with increased levels of the antioxidant chlorogenic acid. Nat. Biotechnol. 22:746–754.

Norizan, S. N. M., Yin, W.-F., and Chan, K.-G. 2013. Caffeine as a potential quorum sensing inhibitor. Sensors (Basel). 13:5117–5129.

O'May, C., and Tufenkji, N. 2011. The swarming motility of *Pseudomonas aeruginosa* is blocked by cranberry proanthocyanidins and other tannin-containing materials. Appl. Environ. Microbiol. 77:3061–3067.

Osbourn, A. E. 2003. Saponins in cereals. Phytochemistry. 62:1–4.

Ouattara, B., Simard, R. E., Holley, R. A., Piette, G. J.-P., and Bégin, A. 1997. Antibacterial activity of selected fatty acids and essential oils against six meat spoilage organisms. Int. J. Food Microbiol. 37:155–162.

Oura, H., Tashiro, Y., Toyofuku, M., Ueda, K., Kiyokawa, T., Ito, S., et al. 2015. Inhibition of *Pseudomonas aeruginosa* swarming motility by 1-naphthol and other bicyclic compounds bearing hydroxyl groups. Appl. Environ. Microbiol. 81:2808–2818.

Paczkowski, J. E., Mukherjee, S., McCready, A. R., Cong, J.-P., Aquino, C. J., Kim, H., et al. 2017. Flavonoids Suppress *Pseudomonas aeruginosa* virulence through allosteric inhibition of quorum-sensing receptors. J. Biol. Chem. 292:4064–4076.

Paczkowski, J. E., Mukherjee, S., McCready, A. R., Cong, J.-P., Aquino, C. J., Kim, H., et al. 2017. Flavonoids suppress *Pseudomonas aeruginosa* virulence through allosteric inhibition of quorum-sensing receptors. J. Biol. Chem. 292:4064–4076.

Pandit, S., Cai, J. N., Song, K. Y., and Jeon, J. G. 2015. Identification of anti-biofilm components in *Withania somnifera* and their effect on virulence of *Streptococcus* mutans biofilms. J. Appl. Microbiol. 119:571–581.

Panopoulos, N. J., and Schroth, M. N. 1974. Role of flagellar motility in invasion of bean leaves by *Pseudomonas phaseolicola*. Phytopathology. 64:1389-1397.

Papenfort K, Bassler BL. 2016. Quorum sensing signal-response systems in Gram-negative bacteria. Nat Rev Micro. 14:576-88

Papkou, A., Hedge, J., Kapel, N., Young, B., and MacLean, R. C. 2020. Efflux pump activity potentiates the evolution of antibiotic resistance across *S. aureus* isolates. Nat. Commun. 11:3970.

Paré, P. W., and Tumlinson, J. H. 1999. Plant volatiles as a defense against insect herbivores. Plant Physiol. 121:325–332.

Park, J., Kaufmann, G. F., Bowen, J. P., Arbiser, J. L., and Janda, K. D. 2008. Solenopsin A, a venom alkaloid from the fire ant *solenopsis invicta*, inhibits quorum-sensing signaling in *Pseudomonas aeruginosa*. J. Infect Dis. 198:1198–1201.

Park, J., Kaufmann, G. F., Bowen, J. P., Arbiser, J. L., and Janda, K. D. 2008. Solenopsin A, a venom alkaloid from the fire ant *Solenopsis invicta*, inhibits quorum-sensing signaling in *Pseudomonas aeruginosa*. J Infect Dis. 198:1198–1201.

Parsek, M. R., and Greenberg, E. P. 2005. Sociomicrobiology: the connections between quorum sensing and biofilms. Trends Microbiol. 13:27–33.

Pattnaik, S., Barik, S., Muralitharan, G., and Busi, S. 2018. Ferulic acid encapsulated chitosan-tripolyphosphate nanoparticles attenuate quorum sensing regulated virulence and biofilm formation in *Pseudomonas aeruginosa* PAO1. IET Nanobiotechnol. 12:1056–1061.

Pedras, M. S. C., Yaya, E. E., and Glawischnig, E. 2011. The phytoalexins from cultivated and wild crucifers: Chemistry and biology. Nat. Prod. Rep. 28:1381–1405.

Pereira, D. M., Valentão, P., Pereira, J. A., and Andrade, P. B. 2009. Phenolics: from chemistry to ciology. Molecules. 14:2202–2211.

Perumal, S., Mahmud, R., and Ismail, S. 2017. Mechanism of action of isolated caffeic acid and epicatechin 3-gallate from *Euphorbia hirta* against *Pseudomonas aeruginosa*. Pharmacogn Mag. 13:S311–S315.

Pichersky, E., and Gershenzon, J. 2002. The formation and function of plant volatiles: perfumes for pollinator attraction and defense. Curr. Opin. Plant Biol. 5:237–243.

Pichersky, E., and Raguso, R. A. 2018. Why do plants produce so many terpenoid compounds? New Phytologist. 220:692–702.

Pollumaa L, Alamae T, Mae A. 2012. Quorum sensing and expression of virulence in pectobacteria. Sensors (Basel). 123:3327-49

Prithiviraj B, Bais HP, Weir T, Suresh B, Najarro EH, et al. 2005. Down regulation of virulence factors of *Pseudomonas aeruginosa* by salicylic acid attenuates its virulence on *Arabidopsis thaliana* and *Caenorhabditis elegans*. Infect Immun. 73:5319-28

Rabin, N., Zheng, Y., Opoku-Temeng, C., Du, Y., Bonsu, E., and Sintim, H. O. 2015. Agents that inhibit bacterial biofilm formation. Future Med. Che. 7:647–671.

Rafiq R., Hayek S., Anyanwu U., Hardy B., Giddings V., Ibrahim S., Tahergorabi R., Kang H. 2016. Antibacterial and antioxidant activities of essential oils from *Artemisia herba-alba* Asso., *Pelargonium capitatum* radens and *Laurus nobilis* L. Food*s.* 5:28.

Rahmani-Badi, A., Sepehr, S., Mohammadi, P., Soudi, M. R., Babaie-Naiej, H., and Fallahi, H. 2014. A combination of cis-2-decenoic acid and antibiotics eradicates pre-established catheter-associated biofilms. J. Med. Microbiol. 63:1509–1516.

Ramanathan, S., Ravindran, D., Arunachalam, K., and Arumugam, V. R. 2018. Inhibition of quorum sensing-dependent biofilm and virulence genes expression in environmental pathogen *Serratia marcescens* by petroselinic acid. Antonie van Leeuwenhoek. 111:501–515.

Ramanathan, S., Ravindran, D., Arunachalam, K., and Arumugam, V. R. 2018. Inhibition of quorum sensing-dependent biofilm and virulence genes expression in environmental pathogen *Serratia marcescens* by *petroselinic acid.* Antonie van Leeuwenhoek. 111:501–515.

Rodríguez-Concepción, M. 2006. Early steps in isoprenoid biosynthesis: multilevel regulation of the supply of common precursors in Plant Cells. Phytochem Rev. 5:1–15.

Rudrappa, T., and Bais, H. P. 2008. Curcumin, a known phenolic from Curcuma longa, attenuates the virulence of *Pseudomonas aeruginosa* PAO1 in whole plant and animal pathogenicity models. J. Agric. Food Chem. 56:1955–1962.

Rutherford, S., and Bassler, B. 2012. Bacterial quorum sensing: its role in virulence and possibilities for its control. Cold Spring Harb perspect Med. 2.

Ruwandeepika HA, Karunasagar I, Bossier P, Defoirdt T. 2015. Expression and quorum sensing regulation of type III secretion system genes of *Vibrio harveyi* during infection of gnotobiotic brine shrimp. PLoS One. 10:e0143935

Salini, R., Sindhulakshmi, M., Poongothai, T., and Pandian, S. K. 2015. Inhibition of quorum sensing mediated biofilm development and virulence in uropathogens by *Hyptis suaveolens*. Antonie van Leeuwenhoek. 107:1095–1106.

Santhakumari, S., Nilofernisha, N. M., Ponraj, J. G., Pandian, S. K., and Ravi, A. V. 2017. In vitro and in vivo exploration of palmitic acid from *Synechococcus elongatus* as an antibiofilm agent on the survival of *Artemia franciscana* against virulent vibrios. J. Invertebr. Pathol. 150:21–31.

Sepehr, S., Rahmani-Badi, A., Babaie-Naiej, H., and Soudi, M. R. 2014. Unsaturated fatty acid, cis-2-Decenoic Acid, in combination with disinfectants or antibiotics removes pre-established biofilms formed by food-related bacteria. PLOS ONE. 9:e101677.

Sethupathy, S., Vigneshwari, L., Valliammai, A., Balamurugan, K., and Karutha Pandian, S. 2017. l -Ascorbyl 2,6-dipalmitate inhibits biofilm formation and virulence in methicillin-resistant Staphylococcus aureus and prevents triacylglyceride accumulation in *Caenorhabditis elegans*. RSC Adv. 7:23392–23406.

Sharma, A., Gupta, V. K., and Pathania, R. 2019. Efflux pump inhibitors for bacterial pathogens: from bench to bedside. IJMR. 149:129–145.

Shen, Y., Jin, L., Xiao, P., Lu, Y., and Bao, J. 2009. Total phenolics, flavonoids, antioxidant capacity in rice grain and their relations to grain color, size and weight. J. Cereal Sci. 49:106–111.

Shrigod N.M., Swami Hulle N.R., Prasad R.V. 2017. Supercritical fluid extraction of essential oil from mint leaves (*Mentha spicata*): Process optimization and its quality evaluation. J. Food Process Eng. 40:12488.

Shukla, V., and Bhathena, Z. 2016. Broad spectrum anti-quorum sensing activity of tannin-rich crude extracts of Indian medicinal plants. Scientifica. 2016:5823013.

Silhavy, T. J., Kahne, D., and Walker, S. 2010. The Bacterial cell envelope. Cold Spring Harb Perspect Biol. 2:a000414.

Silverman, J. M., Brunet, Y. R., Cascales, E., and Mougous, J. D. 2012. Structure and regulation of the type VI secretion system. Annu. Rev. Microbiol. 66:453–472.

Simionato, A. V. C., Silva, D. S. da, Lambais, M. R., and Carrilho, E. 2007. Characterization of a putative Xylella fastidiosa diffusible signal factor by HRGC-EI-MS. J. Mass Spectrom. 42:490–496.

Singh, V. K., Kavita, K., Prabhakaran, R., and Jha, B. 2013. Cis-9-octadecenoic acid from the rhizospheric bacterium *Stenotrophomonas maltophilia* BJ01 shows quorum quenching and anti-biofilm activities. Biofouling. 29:855–867.

Souza, E. L., Oliveira, C. E. V., Stamford, T. L. M., Conceição, M. L., and Gomes Neto, N. J. 2013. Influence of carvacrol and thymol on the physiological attributes, enterotoxin production and surface characteristics of *Staphylococcus aureus* strains isolated from foods. Braz. J. Micriobiol. 44:29–36.

Steindler, L., Bertani, I., De Sordi, L., Schwager, S., Eberl, L., and Venturi, V. 2009. LasI/R and RhlI/R quorum sensing in a strain of *Pseudomonas aeruginosa* beneficial to plants. AEM. 75:5131–5140.

Stenz, L., François, P., Fischer, A., Huyghe, A., Tangomo, M., Hernandez, D., et al. 2008. Impact of oleic acid (cis-9-octadecenoic acid) on bacterial viability and biofilm production in *Staphylococcus aureus*. FEMS Microbiol. Lett. 287:149–155.

T, R., and Hp, B. 2008. Curcumin, a known phenolic from Curcuma longa, attenuates the virulence of *Pseudomonas aeruginosa* PAO1 in whole plant and animal pathogenicity models. J. Agric. Food Chem. 56:1955–1962.

Tapia-Rodriguez, M. R., Hernandez-Mendoza, A., Gonzalez-Aguilar, G. A., Martinez-Tellez, M. A., Martins, C. M., and Ayala-Zavala, J. F. 2017. Carvacrol as potential quorum sensing inhibitor of *Pseudomonas aeruginosa* and biofilm production on stainless steel surfaces. Food Control. 75:255–261.

Teper, D., Zhang, Y., and Wang, N. 2019. TfmR, a novel TetR-family transcriptional regulator, modulates the virulence of *Xanthomonas citri* in response to fatty acids. Mol. Plant Pathol. 20:701–715.

Truchado, P., Giménez-Bastida, J.-A., Larrosa, M., Castro-Ibáñez, I., Espín, J. C., Tomás-Barberán, F. A., et al. 2012. Inhibition of quorum sensing (QS) in *Yersinia enterocolitica* by an orange extract rich in glycosylated flavanones. J. Agric. Food Chem. 60:8885–8894.

Truchado, P., Tomás-Barberán, F. A., Larrosa, M., and Allende, A. 2012. Food phytochemicals act as quorum sensing inhibitors reducing production and/or degrading autoinducers of *Yersinia enterocolitica* and *Erwinia carotovora*. Food Control. 24:78–85.

Tsao, R., Khanizadeh, S., and Dale, A. 2006. Designer fruits and vegetables with enriched phytochemicals for human health. Can. J. Plant Sci. 86:773–786.

Vandeputte OM, Kiendrebeogo M, Rajaonson S, Diallo B, Mol A, et al. 2010. Identification of catechin as one of the flavonoids from Combretum albiflorum bark extract that reduces the production of quorum-sensing-controlled virulence factors in *Pseudomonas aeruginosa* PAO1. Appl. Environ. Microbiol. 76:243-53

VanEtten, H. D., Mansfield, J. W., Bailey, J. A., and Farmer, E. E. 1994. Two classes of plant antibiotics: phytoalexins versus "phytoanticipins." Plant Cell. 6:1191–1192.

Vasireddy L., Bingle L.E.H., Davies M.S. 2018. Antimicrobial activity of essential oils against multidrug-resistant clinical isolates of the *Burkholderia cepacia* complex. PLoS ONE. 13.

Vikram, A., Jesudhasan, P. R., Jayaprakasha, G. K., Pillai, B. S., and Patil, B. S. 2010. Grapefruit bioactive limonoids modulate *E. coli* O157:H7 TTSS and biofilm. Int. J. Food Microbiol. 140:109–116.

Vilariño, M. del P., and Ravetta, D. A. 2008. Tolerance to herbivory in lupin genotypes with different alkaloid concentration: Interspecific differences between *Lupinus albus* L. and *Angustifolius* L. Environ. Exp. Bot. 63:130–136.

Vranová, E., Coman, D., and Gruissem, W. 2013. Network analysis of the MVA and MEP pathways for isoprenoid synthesis. Annu. Rev. Plant Biol. 64:665–700.

Wang, C.-Y., Chen, Y.-W., and Hou, C.-Y. 2019. Antioxidant and antibacterial activity of seven predominant terpenoids. Int. J. Food Prop. 22:230–238.

Wang, H., Chu, W., Ye, C., Gaëta, B., Tao, H., Wang, M., et al. 2019. Chlorogenic acid attenuates virulence factors and pathogenicity of *Pseudomonas aeruginosa* by regulating quorum sensing. Appl. Microbiol. Biotechnol. 103:903-915

Wei, Y., Perez, L. J., Ng, W.-L., Semmelhack, M. F., and Bassler, B. L. 2011. Mechanism of *Vibrio cholerae* autoinducer-1 biosynthesis. ACS Chem. Biol. 6:356–365.

Wenderska, I. B., Chong, M., McNulty, J., Wright, G. D., and Burrows, L. L. 2011. Palmitoyl-DL-carnitine is a multitarget inhibitor of *Pseudomonas aeruginosa* biofilm development. Chembiochem. 12:2759–2766.

Yamazaki A, Li J, Zeng Q, Khokhani D, Hutchins WC, et al. 2012. Derivatives of plant phenolic compound affect the type III secretion system of *Pseudomonas aeruginosa* via a GacS-GacA two component signal transduction system. Antimicrob. Agents Chemother. 56:36-43

Yang, C., Cui, C., Ye, Q., Kan, J., Fu, S., Song, S., et al. 2017. *Burkholderia cenocepacia* integrates cis-2-dodecenoic acid and cyclic dimeric guanosine monophosphate signals to control virulence. PNAS. 114:13006–13011.

Yang, J., Tauschek, M., and Robins-Browne, R. M. 2011. Control of bacterial virulence by AraC-like regulators that respond to chemical signals. Trends in Microbiol. 19:128–135.

Yang, L., and Stöckigt, J. 2010. Trends for diverse production strategies of plant medicinal alkaloids. Nat. Prod. Rep. 27:1469–1479.

Yang, L., Browning, J. D., and Awika, J. M. 2009. Sorghum 3-deoxyanthocyanins possess strong phase II enzyme inducer activity and cancer cell growth inhibition properties. J. Agric. Food Chem. 57:1797–1804.

Zender, M., Klein, T., Henn, C., Kirsch, B., Maurer, C. K., Kail, D., et al. 2013. Discovery and biophysical characterization of 2-Amino-oxadiazoles as novel antagonists of PqsR, an important regulator of *Pseudomonas aeruginosa* Virulence. J. Med. Chem. 56:6761–6774.

Zrira S. and Ghanmi M. 2016. Chemical composition and antibacterial activity of the essential of *Cedrus atlantica* (Cedarwood oil). J. Essent. Oil-Bearing Plants. 19:1267–1272.

Zulak, K. G., and Bohlmann, J. 2010. Terpenoid biosynthesis and specialized vascular cells of conifer defense. J. Integr. Plant. Biol. 52:86–97.

CHAPTER II: DEVELOPMENT OF AUTOMATED PRIMER DESIGN WORKFLOW UNIQPRIMER AND DIAGNOSTIC PRIMERS FOR THE BROAD HOST RANGE PLANT PATHOGEN *DICKEYA DIANTHICOLA*[1][2]

Synopsis

Uniqprimer, a software pipeline developed in Python, was deployed as a user-friendly internet tool in Rice Galaxy for comparative genome analysis to design primer sets for PCR assays capable of detecting target bacterial taxa. The pipeline was trialed with *Dickeya dianthicola*, a destructive broad-host range bacterial pathogen found in most potato-growing regions. *Dickeya* is a highly variable genus, and most primers reported to detect this genus and species exhibit common diagnostic failures. Upon uploading a selection of target and non-target genomes, six primer sets were rapidly identified with Uniqprimer, of which two were specific and sensitive when tested with *D. dianthicola*. The remaining four amplified a minority of the non-target strains tested. The two promising candidate primer sets were trialed with DNA isolated from 116 field samples from across the United States (US) that were previously submitted for testing. *D. dianthicola* was detected in 41 samples demonstrating the applicability of our detection primers and suggesting widespread occurrence of *D. dianthicola* in North America.

1. Introduction

Bacterial plant diseases cause large global financial losses in numerous crop species. Pathogen detection is one of the most important management tools for both endemic and invasive bacterial diseases, allowing growers and regulators to exclude infected planting material and to

[1] This chapter has been published in the Plant Disease Journal.

[2] List of authors: Shaista Karim, Ryan R McNally, Afnan S Nasaruddin, Alexis DeReeper, Ramil P. Maulen, Amy O Charkowski, Jan E Leach, Asa Ben-Hur, Lindsay R Triplett

avoid planting in contaminated soil or irrigating with contaminated water. At state and national borders, bacterial detection is crucial to identifying and quarantining seeds or other plant propagative materials infected with exotic bacterial pathogens. Cost- and time-sensitive decisions about crop destruction or quarantine may hinge on diagnostic test results, so the tests used must be sensitive (*i.e.*, able to detect all strains of the target group), specific (does not produce false positives on non-target groups), and rapid. Widely used molecular tools for bacterial phytopathogen detection include Enzyme Linked Immunosorbent Assay (ELISA), Polymerase Chain Reaction (PCR), isothermal detection methods such as recombinase polymerase amplification (RPA) and loop-mediated isothermal amplification (LAMP), or a combination of these methods. Of these, PCR and other DNA-based methods are preferred for their sensitivity, specificity, and the ease with which the required reagents can be obtained. Thousands of available pathogen genomes have enabled comparative genomic analysis as a means to design PCR-based detection assays, and over a dozen published pipelines or programs have been designed for genomic based differential primer design. Alignment-based pipelines such as KPATH, Insignia, and TOPSI generate and parse alignments of whole genomes or coding sequences to identify signatures unique to the diagnostic targets (Phillippy et al. 2009; Satya et al. 2010; Slezak et al. 2003). Alignment-free approaches, such as RUCS, PrimerHunter, PriMux, and the python package find_differential_primers.py, design primers or probes comprehensively or at intervals across a reference genome before screening them for target specificity across genomes (Hysom et al. 2012; Nelson et al. 2009; Pritchard et al. 2012; Thomsen et al. 2017). While existing programs are designed to solve a variety of different diagnostic needs, adoption of comparative genomics methods for plant disease assay development has still been limited in scope. Many published tools require comfort with a command-line environment, while web tools such as PrimerBLAST or

MgenomeSubtractor depend on genome annotation or are limited to the analysis of few sequences (Shao et al 2010; Ye et al. 2012). Some previously published web tools are no longer maintained (Frech et al. 2009; van Hijum et al. 2003; Phillippy et al. 2009). Together, these factors may have limited the widespread adoption of comparative genomics methods for primer design in bacterial phytopathology. Our goal was to develop a rapid, web-based platform to generate robust diagnostic primers from bacterial sequence data with a minimum of upstream preparation and downstream validation.

We developed an alignment-based primer design pipeline implemented in Python, named Uniqprimer, that combines whole-genome alignment and parsing with primer design and cross-validation. The pipeline shares commonalities with some previously designed programs, particularly the TOPSI package (Satya et al. 2010), in its use of a series of pairwise genome alignments to identify sequences specific to diagnostic targets. Also similarly to other pipelines, Uniqprimer employs the popular tools Primer3 (used in many published pipelines), EMBOSS Primersearch (used by (Pritchard et al. 2012) and BLAST used by (Pritchard et al. 2012; Satya et al. 2010; Thomsen et al. 2017) for primer design and screening steps. However, Uniqprimer uses a strategy distinct from previous alignment-based programs, first isolating amplicon-length regions of sequence that are distinct between target and non-target genomes. This strategy is intended to limit the number of initial primer pairs advanced to primer-genome alignment stages, as well as to enable further user analyses on the isolated regions. In-house versions of this pipeline have previously been used for development of several validated conventional or LAMP primer sets (Ash et al. 2014; Lang et al. 2014; Lang et al. 2017; Langlois et al. 2017; Triplett et al. 2011; Triplett et al. 2015). Recently, we released an online version of Uniqprimer, as one of many tools on the Rice Galaxy resource (galaxy.irri.org, Juanillas et al. 2018) hosted by the International Rice

Research Institute. Galaxy is a user-friendly internet platform that facilitates performing bioinformatics tasks in a reproducible way (Goecks et al. 2010), and workflows can be performed by anyone with a laptop and an internet connection. Although Uniqprimer on Rice Galaxy was reported in a recent paper (Gigascience, in press), its parameters and wet-lab validation have not been previously reported.

Here we describe the Rice Galaxy implementation of the Uniqprimer pipeline and use it to develop diagnostic tools for *Dickeya dianthicola*, a broad host-range pathogen responsible for a recent outbreak of blackleg in potato in North America (Ma et al. 2018). *D. dianthicola* is a pectolytic Gram-negative bacterial pathogen that colonizes potato stems and tubers and causes stem blackening, plant wilt, and tuber decay in potato (Charkowski 2018). Dozens of *Dickeya* sequences are currently available, as well as numerous genome sequences from closely related species (Zhang et al. 2016). Primer development for *Dickeya* species, however, has been continually problematic due to its wide genomic diversity and frequent exchanges. In 2013, Pritchard et al. used a comprehensive alignment-free pipeline to design more than 80 primers for *Dickeya* detection, of which DIA-A and DIA-C were successfully adopted for widespread diagnostic use. A primer set that was previously reliable, DIA-A, was found to yield false negatives on a subset of recently described North American *D. dianthicola* strains that had lost the target operon for these primers (A. O. Charkowski and N. T. Perna, unpublished data). Recently designed primers based on the arbitrarily chosen *dnaX* failed when tested with field samples (van der Wolf et al. 2014). Therefore, the North American potato industry is currently dependent on a single primer set, DIA-C, for diagnosis and detection of this highly variable pathogen, which is likely to result in selection for strains that lack this target sequence. This is a common problem in all genome-based design assays and highlights the need to have multiple distinct detection tools at

hand. To provide additional assays for the potato industry, we used Uniqprimer to develop additional primer sets that can be used in specific and sensitive assays for *D. dianthicola* detection and disease management.

2. Materials and Methods

2.1. Uniqprimer design and implementation on Rice Galaxy

Uniqprimer is a Python script that can be run as a stand-alone script in a Linux command line environment. Uniqprimer incorporates whole-genome alignment and parsing with primer design tools and *in silico* cross-validation. The Uniqprimer Python code is hosted at the Southgreen github repository (https://github.com/SouthGreenPlatform/Uniqprimer). A functional Uniqprimer tool is publicly available via the open-source bioinformatics internet portal Galaxy (Afgan et al. 2018). The graphical interface of Uniqprimer in Rice Galaxy accepts inputs of "include" (diagnostic target) genomes and "exclude" (diagnostic non-target) genomes, which are uploaded by the user as single or multiFASTA sequence format files. The script uses NUCmer, a suffix tree-based nucleotide alignment program in the MUMmer package to rapidly align one of the target genomes against the combined non-target sequences (Delcher et al. 2002). Regions of the target genome identified as mismatches to all non-target genomes are then aligned to the additional target genomes in iterative fashion, and regions that are not identified as matches in all target genomes are eliminated. Regions that are both distinct to and conserved among the group of target genomes are parsed into a multiFASTA sequence file. This file is used as input for primer design using the Primer3 program (Koressaar and Remm 2007). Primer3 is set at default stringency; users can modify primer length and product size range as desired. Finally, primers are mapped to target and non-target genomes using the Primersearch program from the package EMBOSS (Rice et al. 2000), eliminating any primers that are predicted to amplify a non-target genome or that do not

perfectly match a target genome. The output is a file of primer pair sequences, including predicted product length and melting temperature (T_m), and a logfile with details of the run.

2.2. Primer design and in silico analyses

Uniqprimer was used for the design of primers specific to *D. dianthicola*. To identify relevant genomes for successful primer design, 39 complete or draft genomes representing *Dickeya*, *Pectobacterium* and *Brennaria* isolates were collected from the ASAP on-line database (https://asap.genetics.wisc.edu) and subjected to whole-genome phylogenetic analysis using REALPHY 1.12 (Bertels et al. 2014) (Table 1; Fig. 2). Six *D. dianthicola* genomes, including two draft contig assemblies, were uploaded as target genomes, and 33 other enterobacterial genomes were concatenated and uploaded as non-target genomes (Table 1). All primer options were left on the default settings (product size range 100-300 bp, optimal primer size 20 nucleotides, minimum primer size 18 nucleotides, and max primer size 27 nucleotides). The option to cross-check primers for specificity was selected. Primer output was checked for specificity to *D. dianthicola* among the Genbank nr and Refseq representative genomes databases using Primer-BLAST (Ye et al. 2012). *D. dianthicola* target genes identified through the Primer-BLAST were analyzed for conservation among *Dickeya* and *Pectobacterium* using BlastN (Altschul et al. 1990).

2.3. Source and identity of isolates and environmental samples

Bacterial strains used in this study are listed in Table 1. The collection included multiple *D. dianthicola* strains, *Dickeya* species, genera related to *Dickeya*, and other bacteria found on potato. The bacterial strains were identified through morphology and either multi-locus sequence or genome sequence analyses. To confirm the specificity of our primers on diverse agricultural samples, DNA isolated from potato tissues samples and irrigation water was collected from

multiple US states including California, Colorado, Florida, Michigan, Missouri, New Mexico, New York, North Carolina, Texas, and Wisconsin.

2.4. Bacterial DNA extraction

Bacterial isolates were streaked onto nutrient agar and were grown at 25°C for two days (Schaad et al. 2001). Bacterial cells were harvested from the plate and DNA was extracted using a FastPrep kit (MP Biomedicals). To extract bacterial DNA from plant stems and tubers, a tissue sample was excised from the border of the symptomatic regions. The sample was vortexed in 400 µl of sterile water and DNA was extracted from the water with a FastDNA Spin Kit for Soil (MP Biomedicals). DNA quality and quantity was determined for DNA isolated from pure cultures and from diagnostic samples with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). DNA was stored at -20°C.

2.5. Specificity of cPCR and qPCR assays

Purified bacterial genomic DNA was serially diluted in sterile water to concentrations ranging from 1 ng to 100 fg, and these standards were used to determine the sensitivity of conventional (cPCR) and real-time (qPCR) assays. For cPCR, assays were considered positive if a band was visible by imaging on a 2.0% agarose gel after staining with SYBR safe DNA gel stain (Thermo Fisher Scientific) and for qPCR, we used a C_T value of 28.22±2 as indication of *D. dianthicola* detection.

For primer set DDI-1, PCR was conducted in a 25 µl reaction that contained 10 µl of Q-solution (5x), 1 µl of dNTP mix (10 mM), 5 µl of reaction buffer (10x), 1 µl of DDI-1 primers (10 µM), 10 ng of DNA template, and 0.5 µl of HotstartTaq DNA polymerase (5 units/µl) (Table 2). Thermal cycling parameters consisted of 3 minutes at 95°C, 30 seconds at 95°C, followed by 25 cycles of 30 seconds at 57°C, incubation for 5 minutes at 72°C and 10°C for infinite hold.

The specificity of primer set DDI-2 was evaluated using the same bacterial isolate collection as used for cPCR testing. For qPCR, the reactions were conducted in 20 µl, including 1 µl primers (10 µM), 2 µl of the DNA template (50 ng), and 10 µl of Power SYBR Green PCR master mix (2x) (Applied Biosystems) (Table 2). The thermal cycling conditions were: 2 min and 30 sec at 98°C, followed by 30 cycles of 10 sec for 98°C, and 1 min at 60°C in an ABI-700 real-time PCR system (Applied Biosystems). The threshold cycles were analyzed and calculated based on absolute quantification, and values less than 24±3 were considered as a positive detection response.

To validate the ability of qPCR to detect *D. dianthicola* in field samples, potato tubers and stems exhibiting blackleg and soft rot symptoms were collected from farmers throughout the US. Tissue samples were suspended in 10 ml sterile water and DNA was extracted using FastDNA Spin Kit for Soil (MP Biomedicals) and stored at -20°C.

2.6. Sensitivity of cPCR and qPCR assays

The sensitivity of primers sets DDI-1 and DDI-2 was evaluated with *D. dianthicola* DNA. From the template set based on pure DNA, a 10-fold series of dilutions was made by diluting a purified genomic DNA solution to concentrations from 1 ng to 100 fg. qPCR and cPCR were conducted with both template sets. All samples were run in three separate reactions. cPCR and qPCR were conducted with both template sets.

3. Results

3.1. Description of the Uniqprimer Rice Galaxy Tool

Uniqprimer in Rice Galaxy is implemented using standard Galaxy tool development practices to wrap the Uniqprimer Python script that executes the primer design pipeline (uniqprimer.py; https://github.com/SouthGreenPlatform/Uniqprimer/tree/master/uniqprimer-

0.5.0). The standalone python script collects as inputs (in the command-line parameters) the following: i) the FASTA files to include (the sequences for which you want to design specific primer), ii) one to several FASTA files to exclude (primers designed will not match these sequences), iii) the product size range (from 100-300 bases default), the optimal primer size (default is 20), the minimum primer size (default = 18) , the maximum primer size (default = 27), and iv) a setting to cross-validate primers by Primersearch alignment to exclude files (default is YES). The Galaxy XML tool file implements the graphical interface to capture these parameters and pass these on to the Uniqprimer python script (uniqprimer.xml, available at https://github.com/SouthGreenPlatform/Uniqprimer).

3.2. Design of D. dianthicola species-specific primers with Uniqprimer

Prior to primer design, 39 genome sequences representing the Pectobacteriaceae were analyzed via whole-genome phylogenetic analysis (Fig. 2). Six genomes were confirmed or predicted to be *D. dianthicola* (Fig. 2). The six confirmed *D. dianthicola* genomes were used as inputs for Uniqprimer analysis with 33 control genomes representing other *Dickeya*, *Pectobacterium* and *Brenneria* species (Table 1; Fig. 2). Uniqprimer analysis was executed in nine minutes and 42 seconds in our instance; execution time may vary according to the number and size of input genomes, the number of initial primers screened, and the number of simultanous users on RiceGalaxy. As part of its output, Uniqprimer identified twenty-four genome regions as diagnostic candidate regions predicted to be distinct to and conserved among *D. dianthicola*. Of the initial primer pairs designed from those regions (two from each region), six primer pairs were confirmed via the Uniqprimer cross-check step to match all target genomes but no excluded genomes provided. Uniqprimer generated an output file of six primer pairs that were designated as DDI-1 through DDI-6, with DDI-1 and DDI-4 originating from the same region and differing only in one

nucleotide in the forward primer (Table 2). PrimerBLAST was used to confirm that all six pairs had strong predicted templates in *D. dianthicola* genomes, but not in any other bacterial genomes found in the RefSeq and nr databases. Mapping primers to target regions in PrimerBLAST, followed by BLASTn searches of the target regions against Genbank, revealed that all of the target genes identified by Uniqprimer have homologs widely distributed among enterobacteria with the exception of the *pehW* locus (Table 2). Primers DDI-1 and DDI-4 targeted the same region of heme-binding ABC transporter *ddpA* (WP_024104074). Primers DDI-3 and DDI-5 targeted different regions of the *coaABCD* locus involved in pantetheine 4'-phosphate synthesis. These results suggest that *ddpA* and *coaABCD* could represent regions of particular diagnostic value in the Pectobacteriaceae. No regions present only in *Dickeya* or present only in the Pectobacteriaceae were identified as primer targets by Uniqprimer.

3.3. Sensitivity and specificity of cPCR and qPCR assays

DDI primer sets identified by Uniqprimer were tested with purified DNA from *D. dianthicola* and related species. DDI-3, DDI-4 and DDI-6 primer sets amplified DNA from non-target species in addition to *D. dianthicola* and thus were excluded from subsequent analysis (Table 2). In addition, DDI-6, predicted to anneal to the *pehW* locus, did not reliably amplify DNA from all *D. dianthicola* strains analyzed (Table 2). Primer sets DDI-1 and DDI-2 were sensitive to all strains of *D. dianthicola*, and no amplification was detected from other non-*D. dianthicola* species (Fig. 3; Fig. 4; Table 2). Using primers DDI-1 and DDI-2, neither cPCR nor qPCR amplified non-target DNA from any of the other plant- and soil-associated bacteria that were tested, including the soft rot bacterial pathogen *Pectobacterium* and the closely related plant pathogenic genera *Brenneria* and *Erwinia* (Fig. 3).

3.4. Detection limit of cPCR and qPCR assays

DDI-1 and DDI-2 were tested to confirm detection limit with known *D. dianthicola* genomic DNA and field samples. To prepare for sensitivity testing, *D. dianthicola* primers were tested to confirm efficiency. Both DDI-1 and DDI-2 exhibited efficiency values within accepted limits (100±10%). To determine detection limits for our assays, 100 ng of template DNA was serially diluted to one pg and tested via cPCR and qPCR. cPCR assays with DDI-1 detected *D. dianthicola* DNA at 10 pg. qPCR assays with DDI-2 exhibited detection limits down to 1 pg of DNA template. DDI-1, DDI-2, and DIA-C consistently identified *D. dianthicola* in field samples, while DIA-A, pelADE, and Df/Dr were inconsistent in identification from field samples, suggesting poor specificity (Table 3; Fig. 4).

3.5. Field sample analysis

To confirm the utility of our *D. dianthicola* detection primers and to further demonstrate their specificity, we validated our Uniqprimer assays with DNA from field samples collected across the United States. In total, 52 potato stem samples, 14 potato tuber samples, 9 potato tissue culture samples, and 41 irrigation water samples were tested from California, Colorado, Florida, Michigan, Missouri, New Mexico, New York, North Carolina, Texas, and Wisconsin (Table 3). *D. dianthicola* was detected in 30 stem and 11 tuber samples from California, Florida, Michigan, Montana, New Mexico, New York, North Carolina, Texas and Wisconsin. We compared the results with previously published assays and our results were consistent with DIA-C *D. dianthicola* detection primers (Pritchard et al. 2013).

3.6. Comparison DDI-1 and DDI-2 to extant tools for D. dianthicola detection

Currently, DNA-based *D. dianthicola* detection relies on the use of species-specific primers DIA-A and DIA-C combined with *Dickeya*-general primers pelADE and Df/Dr (Nassar et

al. 1996; Potrykus et al., 2014; Pritchard et al. 2013). We compared our Uniqprimer assays to DIA-A and DIA-C *in silico* using a Primer-BLAST analysis of the NCBI non-redundant whole-genome database (Fig. 7); Primer-BLAST detects potential amplification products in NCBI genomic databases (Ye et al., 2012). DDI-1, DDI-2, and DIA-A shared 100% nucleotide sequence identity with their cognate targets in all available complete *D. dianthicola* genomes (Fig. 7). DDI-1 and DDI-2 also shared a lower level of identity with amplicon-sized regions in a greater number of off-target genomes than DIA-A or DIA-C (Fig. 7, blue shaded regions). Although this level of identity was not sufficient to result in any false positives for DDI-1 or DD1-2 on strains tested in the lab (Table 1), they illustrate that Uniqprimer's output includes diagnostic targets with some level of homology in closely related genomes, rather than targets completely missing in those genomes.

4. Discussion

We used the Rice Galaxy implementation of the Uniqprimer diagnostic primer design pipeline to efficiently develop primers for sensitive and specific detection of *D. dianthicola*, a difficult-to-detect pathogen that causes tuber soft rot and potato blackleg. Given a set of targets and nontarget genome files, Uniqprimer rapidly generated six primer pairs with absolute sensitivity and specificity *in silico*, of which one-third demonstrated absolute sensitivity and specificity against a library of strains in the lab. This lab validation rate is comparable with Pritchard et al (2013). Uniqprimer can be performed by any user with an internet connection and access to genome sequences of diagnostic target bacteria, although the speed of the process may vary according to the size of the dataset input and the number of simultaneous users on RiceGalaxy. While the in-house Python code of Uniqprimer has previously been used to develop primers for specific detection of pathovars and geographic pathogen clades, this work demonstrates the

utilityof the user-friendly internet-accessible implementation of the pipeline for solving difficult diagnostic problems.

The Uniqprimer pipeline is similar to previous primer design approaches focused on the alignment-based identification of target-specific consensus targets, particularly the TOPSI Perl package (Satya et al. 2010), in that the pipeline uses a series of rapid whole-genome alignments to isolate regions that are distinct to and conserved among the diagnostic target strains. However, while other alignment-based programs generate a large number of primer-length "signatures" that are paired into primer sets after screening, Uniqprimer generates a smaller number of amplicon-length primer design templates that share increased conservation among targets than between targets and non-targets. This is in contrast to the computational approach of alignment-free methods, which comprehensively design primer pairs at regular intervals or for all coding sequences prior to filtering. While the goal of this project was not to perform a side-by-side comparison of primer design pipelines, the approach of limiting primer design to a smaller number of highly pre-filtered regions is intended to limit the computationally expensive final screening step of aligning the candidate primers to the input genomes. One benefit of isolating amplicon-length regions is that Uniqprimer output includes multiFASTA files of the candidate diagnostic target regions, i.e., sequences predicted to have distinctions between target and non-target genomes. This output file can be useful for nonconventional primer design, such as primers for LAMP (Ash et al. 2014).

Dickeya is a diverse genus with at least seven known species and additional subspecies with significant genetic overlap and exchange; developing primers for species in this genus has proven difficult. Various immunodetection methods and DNA-based assays are published that describe *D. dianthicola* detection, but most of these assays lack the specificity and the sensitivity

necessary to produce pathogen-free stock. For example, we found that the Df/Dr primer set resulted in false negatives, the pelADE primer set amplified spurious DNA fragments, and the target locus of the DIA-A primer set is missing from some North American strains of *D. dianthicola*. This was unavoidable due to the unavailability of representative genomes from North America during the time of DIA-A primer design (Nassar et al. 1996; Potrykus et al. 2014; Pritchard et al. 2013). Uniqprimer designed two novel primer sets that performed comparably with the industry standard, DIA-C, with DNA from pure bacterial isolates and field samples (Pritchard et al. 2013). The remaining four resulted in false positives on non-target species in the lab, always a possibility when testing on unsequenced strains. The two successful candidate primer pairs were 100% accurate when tested against a wider variety of samples in the lab, demonstrating their strong potential as diagnostic tools. The primer pairs targeted loci that have predicted homologs in multiple genera of enterobacteria, demonstrating the utility of the pipeline for identifying distinct regions of conserved diagnostic targets which may be missed in alignment strategies targeting the presence/absence of entire loci.

To date, no primer sets are available that amplify DNA from all strains within the *Dickeya* genus. We also attempted to use Uniqprimer to design primers for detection of all *Dickeya* species, but the pipeline failed to identify any primer pairs that did not also amplify other pectolytic or enteric bacteria. This suggests that Uniqprimer may require a certain degree of genetic closeness within the diagnostic target group, as well as a threshold of genetic distance between target and non-target groups. Thus, the application of Uniqprimer may be limited for groups genetically broader than the highly specific groups currently validated, including species, pathovar, and sub-pathovar geographic groups (Lang et al. 2014; Langlois et al. 2017). Uniqprimer prioritizes rapid and user-friendly use over exhaustive primer design; previously published strategies using more

comprehensive alignment-free or unique signature approaches may be better suited to develop diagnostics for genus-level detection, high-resolution differentiation of closely related strains, or other difficult diagnostic situations. The use of this pipeline to develop primers both distinct and comparable in performance to *D. dianthicola* primers previously generated through an alignment-free pipeline (Pritchard et al. 2013) illustrates the value of utilizing multiple complementary primer design strategies to maximize diagnostic resources. Sampling for *Dickeya* species has been limited to only a few crops and a few regions in the world, so the discovery of new diversity and the ensuing search for new diagnostics tests will undoubtedly continue.

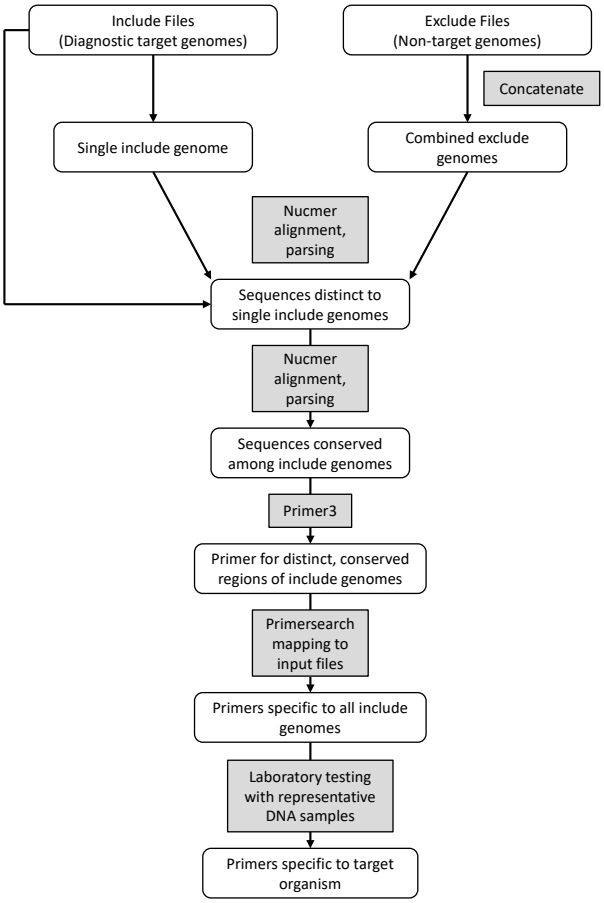


Figure 2.1. Flowchart of the Uniqprimer process for primer design. White boxes represent Uniqprimer stages. Gray boxes represent analytical processes. The bottom two boxes represent manual primer validation steps performed in the lab.

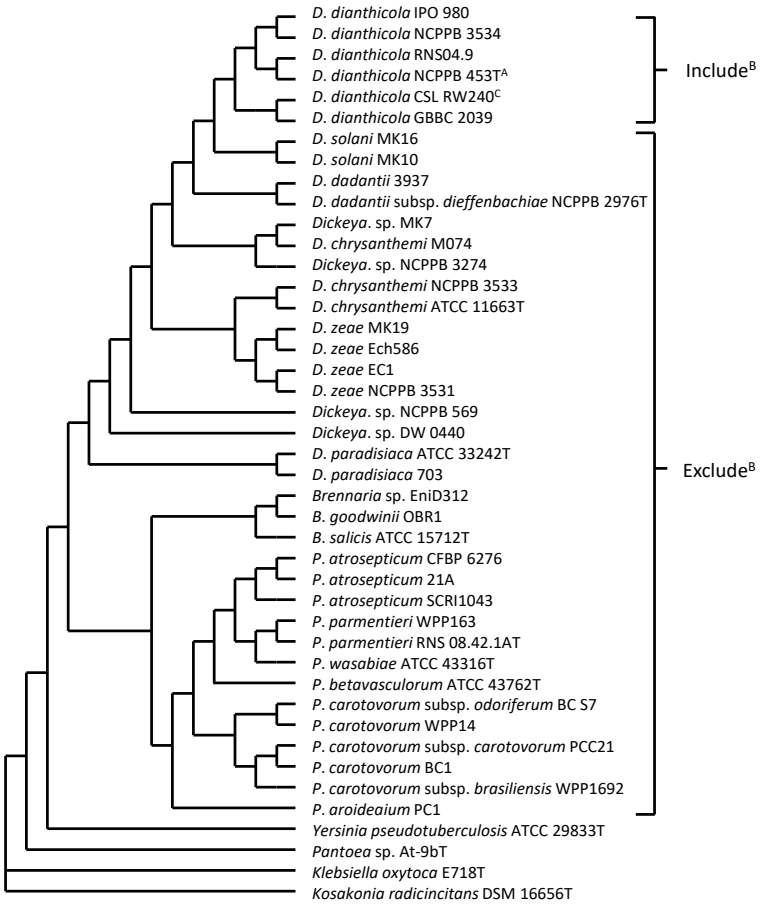


Figure 2.2. Whole-genome phylogeny of Pectobacteriaceae and control strains used for Uniqprimer *D. dianthicola*-specific detection primer design. Tree created with REALPHY 1.12. [A] T = Type strain. [B] Denotes status in Uniqprimer analysis. [C] Previously identified only to genus level.

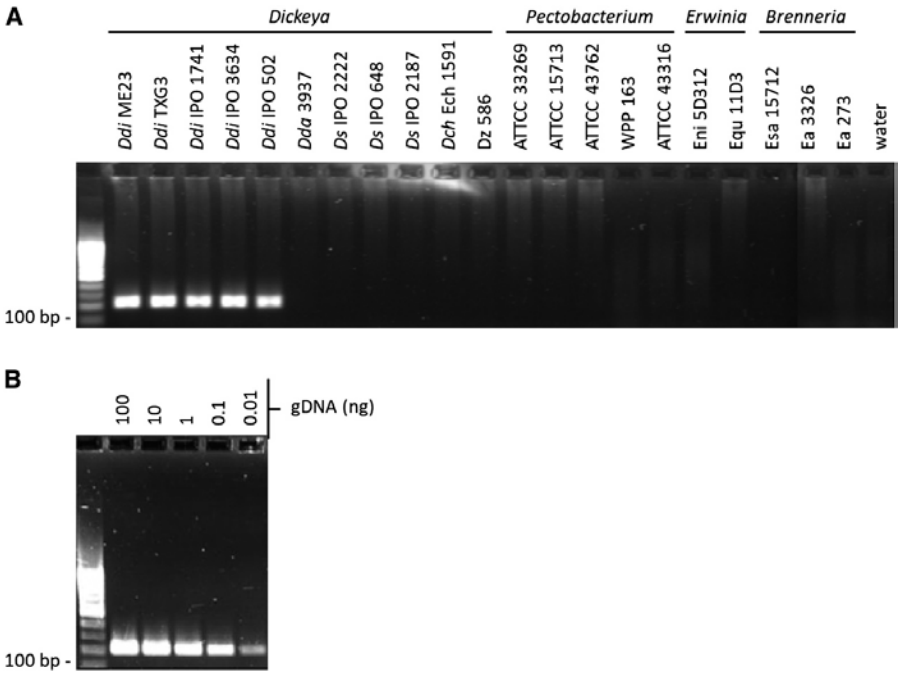


Figure 2.3. Sensitivity and specificity of primers detecting *D. dianthicola* determined through conventional polymerase chain reaction. A, Amplification using DDI-1 detection primers of targeted gene presented by 200-250 bp bands. B, Sensitivity of conventional PCR reaction of pure genomic DNA (gDNA) from *D. dianthicola* ME23.

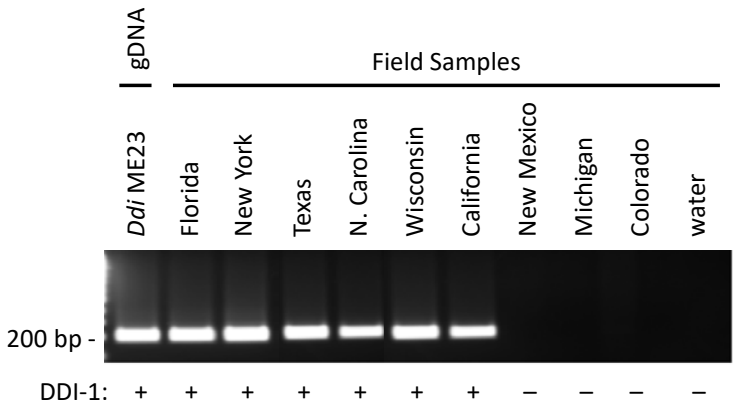


Figure 2.4. Detection of *Dickeya dianthicola* (Ddi) in potato field samples from across the US. genomic bacterial DNA (gDNA) control using DDI-1. + = *D. dianthicola* detected and – = *D. dianthicola* not detected.

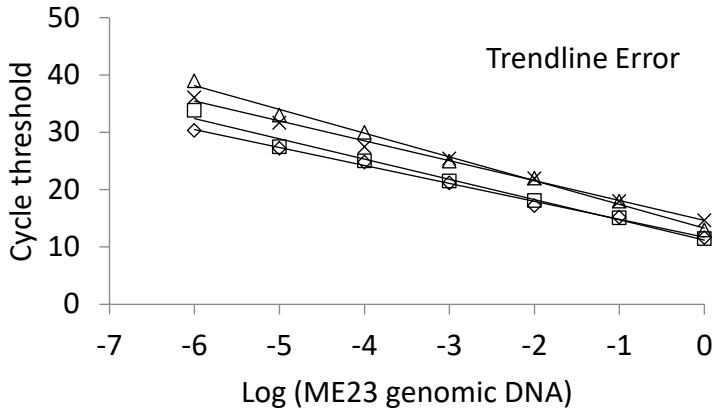


Figure 2.5. Primer efficiencies calculated for quantitative polymerase chain reaction *Dickeya dianthicola* detection primers. DDI-1 (diamond), DDI-2 (square), DIA-A (triangle), and DIA-C (cross) (Pritchard et al. 2013). Results repeated in triplicate using *D. dianthicola* ME23 genomic DNA template. Efficiency calculated [10^(-1/m)-1] × 100.

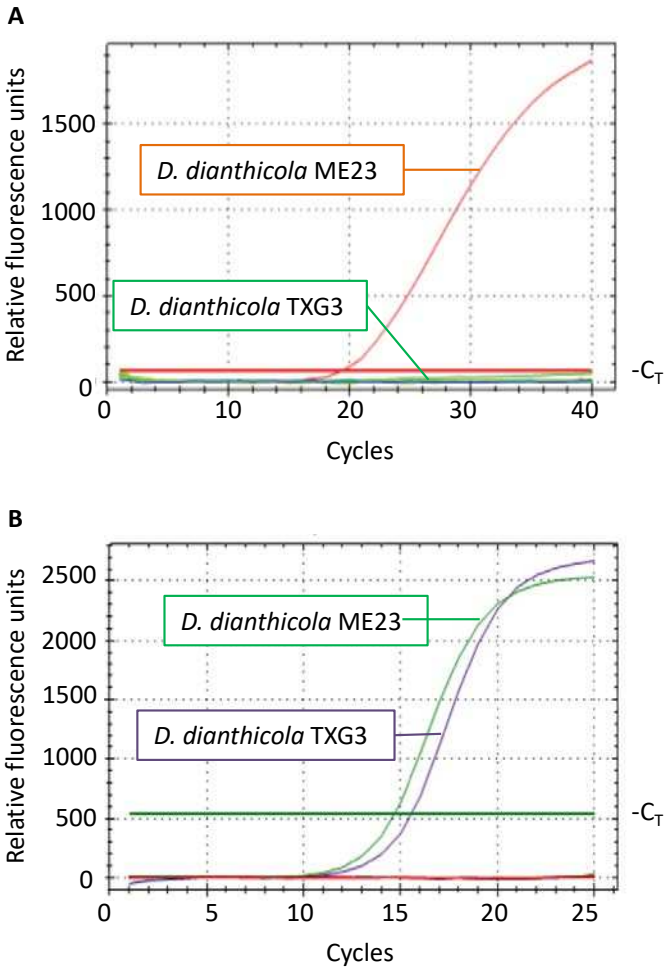


Figure 2.6. Detection of *Dickeya dianthicola* isolates ME23 and TXG3 using quantitative polymerase chain reaction (qPCR). A, qPCR detection of *D. dianthicola* isolates using diagnostic primers DIA-A (Pritchard et al. 2013). B, qPCR detection of *D. dianthicola* isolates using diagnostic primers DDI-2. C_T = cycle threshold as calculated by ABI-700 real-time PCR system.

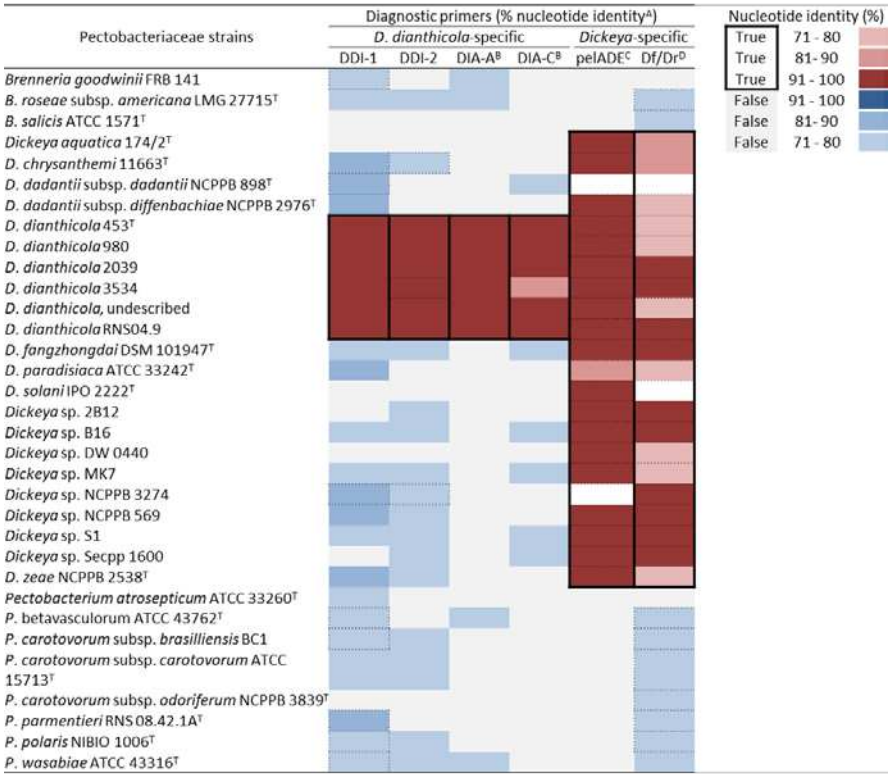


Figure 2.7. In silico analysis of *Pectobacteracease* diagnostic primers and diagnostic primers and predicted targets. A, Nucleotide conservation was detetmind via a Primer3-BLAST analysis and NCBI nonredundant complete genome database. Percent nucleotde identity is presented for true-positive results (red, black border) and false-positive results (blue, grau). Results were compared with primersfrom B, pitchard et al. (2013), C, Nassar et al. (1996) and D, Laurila et al. (2010). Primer-geneome interactions predicted to produce multiple amplicons are noted (dotted border). ^{T} = Type starin.

Table 2.1. Strains and genomes used in this study

Species	Strain	Origin	Host	Uniqprimer[A]	ASAP Genome ID	DDI-1[B]	DDI-2[B]
Brenneria							
B. goodwinii	OBR1	nd[c]	nd	excluded	WIS_BgoOBR1_v1.fas	–	–
B. quercina	Equ 11D3	Beer	Oak	nt[D]	-	–	–
B. salicis	ATCC 15712-T[E]	UK	Willow	excluded	WIS_BsaATCC15712_v1.fas	–	–
Brenneria sp.	Eni D312	nd	Walnut	excluded	WIS_BspEniD312_DRAFTv2.fas	–	–
Dickeya							
D. chrysanthemi	ATCC 11663-T	nd	nd	excluded	WIS_Dch11663_DRAFTv1.fas	–	–
D. chrysanthemi	NCPPB 3533	nd	nd	excluded	WIS_Dch3533_DRAFTv1.fas	–	–
D. chrysanthemi	M074	nd	nd	excluded	WIS_DchM074_DRAFTv1.fas	–	–
D. dadantii	3937	France	African violet	excluded	WIS_ECH3937_v6b.fas	–	–
D. dadantii subsp. dieffenbachiae	NCPPB 2976-T	nd	nd	excluded	WIS_Dda2976_DRAFTv1.fas	–	–
D. dianthicola	GBBC 2039	nd	nd	included	WIS_Ddi2039_DRAFTv1.fas	+	+
D. dianthicola	NCPPB 3534	nd	nd	included	WIS_Ddi3534_DRAFTv1.fas	+	+

	NCPPB 453-						
D. dianthicola	T	nd	nd	included	WIS_Ddi453_DRAFTv1.fas	+	+
D. dianthicola	IPO 980	nd	nd	included	WIS_Ddi980_DRAFTv1.fas	+	+
D. dianthicola	RNS04.9	nd	nd	included	WIS_DdiRNS049_DRAFTv1.fas	+	+
D. dianthicola	undescribed	nd	nd	included	WIS_DicCSLRW240_DRAFTv1.fas	+	+
D. dianthicola	IPO 1741	Netherlands	Potato	nt	-	+	+
D. dianthicola	IPO 3646	Netherlands	Potato	nt	-	+	+
D. dianthicola	IPO 502	nd	Potato	nt	-	+	+
D. dianthicola	ME 23	US	Potato	nt	-	+	+
D. dianthicola	TXG 3	US	Potato	nt	-	+	+
D. paradisiaca	703	nd	nd	excluded	WIS_Dic703_v3.fas	–	–
	ATCC						
D. paradisiaca	33242-T	nd	nd	excluded	WIS_Dpa2511_DRAFTv1.fas	–	–
D. solani	MK10	nd	nd	excluded	WIS_DsoMK10_DRAFTv1.fas	–	–
D. solani	MK16	nd	nd	excluded	WIS_DsoMK16_DRAFTv1.fas	–	–
D. solani	IPO 2222	Netherlands	Potato	nt	-	–	–
D. solani	IPO 2187	Israel	Potato	nt	-	–	–
D. solani	IPO 3648	Netherlands	Potato	nt	-	–	–
Dickeya sp.	NCPPB 3274	nd	nd	excluded	WIS_Dic3274_DRAFTv1.fas	–	–
Dickeya sp.	NCPPB 569	nd	nd	excluded	WIS_Dic569_DRAFTv1.fas	–	–

Dickeya sp.	DW 0440	nd	nd	excluded	WIS_DicDW0440_DRAFTv1.fas	–	–
Dickeya sp.	MK7	nd	nd	excluded	WIS_DicMK7_DRAFTv1.fas	–	–
D. zeae	Ech1591	US	Corn	nt	-	–	–
D. zeae	Ech586	Florida	Philodendron	excluded	WIS_Dic586_v1.fas	–	–
D. zeae	NCPPB 3531	nd	nd	excluded	WIS_Dze3531_DRAFTv1.fas	–	–
D. zeae	EC1	nd	nd	excluded	WIS_DzeEC1_v1.fas	–	–
D. zeae	MK19	nd	nd	excluded	WIS_DzeMK19_DRAFTv1.fas	–	–
Pectobacterium							
P. atrosepticum	21A	nd	nd	excluded	WIS_Pat21A_v1.fas	–	–
P. atrosepticum	CFBP 6276	nd	nd	excluded	WIS_Pat6276_DRAFTv1.fas	–	–
P. atrosepticum	SCRI1043	nd	nd	excluded	WIS_SCRI1043_v2.fas	–	–
P. atrosepticum	ATCC 33260	UK	Potato	nt	-	–	–
P. betavasculorum	ATCC 43762-T	California	Sugar beet	excluded	WIS_Pbe43762_DRAFTv1.fas	–	–
P. betavasculorum	Ecb 2	nd	nd	nt	-	–	–
P. betavasculorum	Ecb 6	nd	nd	nt	-	–	–
P. betavasculorum	Ecb 1	nd	nd	nt	-	–	–
P. carotovorum	WPP14	Wisconsin	Potato	excluded	WIS_EccWPP14_v2.fas	–	–
P. carotovorum	BC1	nd	nd	excluded	WIS_PcaBC1_v1.fas	–	–

	ATTCC						
P. carotovorum	15713	Denmark	Potato	nt	-	–	–
P. carotovorum subsp. *brasiliensis*	WPP1692	nd	nd	excluded	WIS_WPP1692_v2.fas	–	–
P. carotovorum subsp. *brasiliensis*	WPP165	nd	nd	nt	-	–	–
P. carotovorum subsp. *brasiliensis*	WPP5	Wisconsin	Potato	nt	-	–	–
P. carotovorum subsp. *brasiliensis*	WPP20	Wisconsin	Potato	nt	-	–	–
P. carotovorum subsp. *brasiliensis*	WPP1	Wisconsin	Potato	nt	-	–	–
P. carotovorum subsp. *carotovorum*	PCC21	nd	nd	excluded	WIS_PcaPCC21_v1.fas	–	–
P. carotovorum subsp. *carotovorum*	WPP127	Wisconsin	Potato	nt	-	–	–
P. carotovorum subsp. *carotovorum*	WPP156	nd	nd	nt	-	–	–
P. carotovorum subsp. *carotovorum*	WPP2	Wisconsin	Potato	nt	-	–	–

P. carotovorum subsp. *carotovorum*	WPP12	Wisconsin	Potato	nt	-	–	–
P. carotovorum subsp. *carotovorum*	WPP16	Wisconsin	Potato	nt	-	–	–
P. carotovorum subsp. *carotovorum*	WPP17	Wisconsin	Potato	nt	-	–	–
P. carotovorum subsp. *odoriferum*	BC S7	nd	nd	excluded	WIS_PcaBCS7_DRAFTv1.fas	–	–
P. parmentieri	RNS 08.42.1A-T	nd	nd	excluded	WIS_PpaRNS08421A_v1.fas	–	–
P. parmentieri	WPP163	Wisconsin	Potato	excluded	WIS_PwaWPP163_v1.fas	–	–
Pectobacterium sp.	PC1	nd	nd	excluded	WIS_PcaPC1_v2.fas	–	–
P. wasabiae	ATCC 43316-T	Japan	nd	excluded	WIS_Pwa43316_v1.fas	–	–
P. wasabiae	WPP172	Denmark	Potato	nt	-	–	–
P. wasabiae	WPP161	nd	nd	nt	-	–	–
Other							
E. amylovora	Ea 246	nd	Raspberry	nt	-	–	–
E. amylovora	Ea 321	France	Hawthorne	nt	-	–	–

E. amylovora	Ea 273	New York	Apple	nt	-	–	–	

[A] Status of genome in Uniqprimer analysis
[B] Combined results of conventional and quantitative PCR results; '+' = positive for *D. dianthicola* detection; '-' = No *D. dianthicola* detected.
[C] Not determined
[D] Not tested
[E] T = Type strain

Table 2.2. Uniqprimer output of candidate *Dickeya dianthicola* diagnostic primers

Primer	Sequence (5'-3')	Length (bp)	Location[A]	Amplified (#/#)			Source
				Target	Non-target[B]	T_m (°C)[C]	
DDI-F1[D]	CTGACTATGCCTGCGTGAAA	206	4601359-564	5/5	0/36	55-65	This
DDI-R1	CGGAATCAGGCAGAACAGAT		(*dppA*)				publication
DDI-F2 [D]	GTATTCAGCTCCGCCACTTC	201	4654393-593	5/5	0/36	50-65	This
DDI-R2	TTTAACCTGACCAGCGGAGT		(*ubiE*)				publication
DDI-F3	GAACTGCAACTGGCCAAAAT	201	4618221-421	5/5	2/36	nt[E]	This
DDI-R3	AACGACAGGTCGCTTTCAGT		(*coaD*)				publication
DDI-F4	TGACTATGCCTGCGTGAAAC	205	4601359-564	5/5	1/36	nt	This
DDI-R4	CGGAATCAGGCAGAACAGAT		(*dppA*)				publication
DDI-F5	CTGCATCAGGAAATGCGATA	194	4615678-871	5/5	5/36	nt	This
DDI-R5	GTGTTTCCCTGCAAGGTGTT		(*coaBC*)				publication
DDI-F6	CCGCCATACCACAGGTTATC	200	DDI_4012	5/5	3/36	55-65	This
DDI-R6	CAGAGTCGCACCTTTTGACA		(*pehW*)				publication
pelADE1	GATCAGAAAGCCCGCAGCCAGAT	420	3120359-765	nt	nt	-	Nassar et
pelADE2	CTGTGGCCGATCAGGATGGTTTTGTCGTGC		(*pel* cluster)				al. 1996
Df	AGAGTCAAAAGCGTCTTG	130	4674870- 5002	nt	nt	60	Laurila et
Dr	TTTCACCCACCGTCAGTC		(tRNA-Glu)				al. 2010

DIA-A_F	GGCCGCCTGAATACTACATT	100	1154606- 708	nt	nt	59	Pritchard et
DIA-A_R	TGGTATCTCTACGCCCATCA		(oxidoreductase)				al. 2013
DIA-C_F	CCAACGATTAGTCGGATCT	100	1115639-733	nt	nt	59	Pritchard et
DIA-C_R	TAGTTGGTGCCAGGTTGGTA		(reductase)				al. 2013

[A] Location in chromosome of *D. dianthicola* RNS04.9 (GCA_000975305)
[B] Non-target amplification appeared 30 cycles after target amplification.
[C] T_m = melting temperature range at which primers amplified target DNA
[D] Primers presented in this study.
[E] nt; not tested

Table 2.3. Comparison of PCR detection results using *Dickeya dianthicola*-specific and *Dickeya*-general diagnostic primers

Sample type (n)	**Location (n)**	**Pel ADE**			**Df/Dr**			**DIA-A**			**DIA-C**			**DDI-1**			**DDI-2**		
		+[A]	-[B]	*[C]	+	-	*	+	-	*	+	-	*	+	-	*	+	-	*
Stems (52)	California (2)	0	2	0	0	2	0	0	2	0	0	2	0	0	2	0	0	2	0
	Colorado (17)	0	17	2	nt	nt	nt	0	17	0	0	17	0	0	17	0	0	17	0
	Florida (6)	2	0	4	2	0	4	6	0	0	6	0	0	6	0	0	6	0	0
	Missouri (4)	3	0	1	4	0	1	4	0	0	4	0	0	4	0	0	4	0	0
	New Mexico (1)	1	0	0	0	0	2	0	1	0	1	0	0	1	0	0	1	0	0
	New York (5)	0	1	4	1	0	3	4	1	0	4	1	0	4	1	0	4	1	0
	North Carolina (3)	3	0	0	3	nt	nt	3	0	0	3	0	0	3	0	0	3	0	0
	Texas (2)	2	0	0	2	0	0	0	2	0	2	0	0	2	0	0	2	0	0
	Wisconsin (12)	3	5	4	nt	nt	nt	2	10	0	8	4	0	8	4	0	8	4	0
Tubers (14)	Florida (6)	1	0	5	3	2	1	6	0	0	6	0	0	6	0	0	6	0	0
	Texas (2)	0	0	2	2	0	0	0	2	0	2	0	0	2	0	0	2	0	0
	Wisconsin (6)	2	2	6	nt	nt	nt	2	4	0	3	3	0	3	3	0	3	3	0
Tissue culture (9)	Colorado (9)	0	9	0	0	9	0	0	9	0	0	9	0	0	9	0	0	9	0
Water (41)	Colorado (23)	0	23	0	0	20	3	0	23	0	0	23	0	0	23	0	0	23	0
	Michigan (10)	0	10	0	0	10	0	0	10	0	0	10	0	0	10	0	0	10	0
	Wisconsin (8)	0	8	0	nt	nt	nt	0	8	0	0	8	0	0	8	0	0	8	0

[B] -; Negative results
[C] *; Ambiguous results displaying faint or non-specific banding

REFERENCES

Afgan, E., Baker, D., Batut, B., van den Beek, M., Bouvier, D., Čech, M., Chilton, J., Clements, D., Coraor, N., Grüning, B., Guerler, A., Hillman-Jackson, J., Jalili, V., Rasche, H., Soranzo, N., Goecks, J., Taylor, J., Nekrutenko, A., and Blankenberg, D. 2018. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. Nucleic Acids Res. 46:W537-W544.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403-410.

Bertels, F., Silander, O. K., Pachkov, M., Rainey, P. B., and van Nimwegen, E. 2014. Automated reconstruction of whole genome phylogenies from short sequence reads. Mol. Biol. and Evo. 31:1077-1088.

Ash, G.J., Lang, J.M., Triplett, L.R., Stodart, B.J., Verdier, V., Cruz, C. V., Rott, P., and Leach, J. E. 2014. Development of a genomics-based LAMP (Loop-Mediated Isothermal Amplification) assay for detection of *Pseudomonas fuscovaginae* from rice. Plant Dis. 98:909-15.

Charkowski, A. O. 2018. The changing face of bacterial soft-rot diseases. Annu. Rev. Phytopathol. 56:269-288.

Delcher, A.L., Phillippy, A., Carlton, J., and Salzberg, S. L. 2002. Fast algorithms for large-scale genome alignment and comparison. Nucleic Acids Res. 30:2478-83.

Frech, C., Breuer, K., Ronacher, B., Kern, T., Sohn, C., and Gebauer, G. 2009. Hybseek: Pathogen primer design tool for diagnostic multi-analyte assays. Comput. Methods Programs Biomed. 94:152-60.

Goecks, J., Nekrutenko, A., Taylor, J., and Team, T. G. 2010. Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. Genome Biol. 11:R86.

van Hijum, S. A. F. T., de Jong, A., Buist, G., Kok, J., Kuipers, O. P. 2003. UniFrag and GenomePrimer: selection of primers for genome-wide production of unique amplicons. Bioinformatics. 19:1580-1582.

Juanillas, V. M. J, Dereeper, A., Beaume, N., Droc, G., Dizon, J., Mendoza, J. R., Perdon, J. P., Mansueto, L., Triplett, L., Lang, J., Zhou, G., Ratharanjan, K., Plale, B., Haga, J., Leach, J. E. Ruiz, M., Thomson, M., Alexandrov, N., Larmande, P., Kretzschmar, T., and Mauleon, R. P. 2018. Rice Galaxy: an open resource for plant science. bioRxiv. doi: https://doi.org/10.1101/358754

Karim, S., McNally, R. R., Nasaruddin, A. S., DeReeper, A., Mauleon, R. P., Charkowski, A. O., et al. 2019. Development of the Automated Primer Design Workflow Uniqprimer and Diagnostic Primers for the Broad-Host-Range Plant Pathogen *Dickeya dianthicola*. Plant Disease. 103:2893–2902.

Koressaar, T., and Remm, M. 2007. Enhancements and modifications of primer design program Primer3. Bioinformatics 23:1289-91.

Pritchard, L., Holden, N.J., Bielaszewska, M., Karch, H., and Toth, I.K. (2012). Alignment-free design of highly discriminatory diagnostic primer sets for *Escherichia coli* O104:H4 outbreak strains. PloS one 7:e34498.

Lang, J. M., Langlois, P., Nguyen, M. H. R., Purdie, L., Holton, T., Djikeng, A., Cruz, C. M. V., Verdier, V., and Leach, J. E. 2014. Sensitive detection of *Xanthomonas oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* by loop mediated isothermal amplification. Appl. Environ. Microbiol. 80:4519-50

Langlois, P. A., Snelling, J., Hamilton, J. P., Bragard C., Koebnik R., Verdier, V., Triplett, L. R., Blom, J., Tisserat, N. A., and Leach, J. E. 2017. Characterization of the *Xanthomonas translucens* complex using draft genomes, comparative genomics, phylogenetic analysis, and diagnostic LAMP assays. Phytopathology. 107:519-27.

Laurila, J., Hannukkala, A., Nykyri, J., Pasanen, M., Hélias, V., Garlant, L., et al. 2010. Symptoms and yield reduction caused by Dickeya spp. strains isolated from potato and river water in Finland. Eur. J. Plant Pathol. 126:249–262.

Ma, X., Schloop, A., Swingle, B., and Perry, K. L. 2018. *Pectobacterium* and *Dickeya* responsible for potato blackleg disease in New York State in 2016. Plant Dis. 102:1834-40.

Nassar, A., Darrasse, A., Lemattre, M., Kotoujansky, A., Dervin, C., Vedel, R., et al. 1996. Characterization of Erwinia chrysanthemi by pectinolytic isozyme polymorphism and restriction fragment length polymorphism analysis of PCR-amplified fragments of pel genes. Appl Environ Microbiol. 62:2228–2235.

Potrykus, M., Sledz, W., Golanowska, M., Slawiak, M., Binek, A., Motyka, A., et al. 2014. Simultaneous detection of major blackleg and soft rot bacterial pathogens in potato by multiplex polymerase chain reaction. Ann Appl Biol. 165:474–487.

Pritchard, L., Holden, N. J., Bielaszewska, M., Karch, H., and Toth, I. K. 2012. Alignment-free design of highly discriminatory diagnostic primer sets for *Escherichia coli* O104:H4 outbreak strains. PLoS One. 7: e34498.

Pritchard, L., Humphris, S., Saddler, G. S., Parkinson, N. M., Bertrand, V., Elphinstone, J. G., et al. 2013. Detection of phytopathogens of the genus Dickeya using a PCR primer prediction pipeline for draft bacterial genome sequences. Plant Pathol. 62:587–596.

Rice, P., Longden, I., and Bleasby, A. 2000. EMBOSS: the European molecular biology open software suite. Trends Genet. 16:276-7.

Satya, R. V., Kumar, K., Zavaljevski, N., and Reifman, J. 2010. A high-throughput pipeline for the design of real-time PCR signatures. BMC Bioinformatics. 11:340.

Schaad, N. W., Jones, J. B., and Chun, W. 2001. Laboratory Guide for Identification of Plant Pathogenic Bacteria. Third ed. American Phytopathological Society Press. St. Paul, USA.

Shao, Y., He, X., Harrison, E. M., Tai, C., Ou, H-Y., Rajakumar, K., and Deng, Z. 2010. mGenomeSubtractor: a web-based tool for parallel in silico subtractive hybridization analysis of multiple bacterial genomes. Nucleic Acids Res. 38:W194-W200.

Triplett, L. R., Hamilton, J. P., Buell, C. R., Tisserat, N. A., Verdier, V., Zink, F. and Leach, J. E. 2011. Genomic analysis of *Xanthomonas oryzae* isolates from rice grown in the United States reveals substantial divergence from known *X. oryzae* pathovars. Appl. Environ. Microbiol. 77:3930-3937.

Triplett, L. R., Verdier, V., Campillo, T., Van Malderghem, C., Cleenwerck, I., Maes, M., Deblais, L., Corral, R., Koita O., Cottyn, B., and Leach, J. E. 2015. Characterization of a novel clade of *Xanthomonas* isolated from rice leaves in Mali and proposal of *Xanthomonas maliensis* sp. nov. Antonie Van Leeuwenhoek. 107:869-81.

van der Wolf J. M., de Haas, B. H., van Hoof, R., de Haan, E. G., and van den Bovenkamp, G. W. 2014. Development and evaluation of Taqman assays for the differentiation of *Dickeya* (sub)species. Eur. J. Plant Pathol. 138:695-709.

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., and Madden, T. L. 2012. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. BMC Genomics. 13:134-144.

Zhang, Y., Fan, Q., and Loria, R. 2016. A re-evaluation of the taxonomy of phytopathogenic genera *Dickeya* and *Pectobacterium* using whole-genome sequencing data. Syst. Appl. Microbiol. 39:252-259.

Hysom, D.A., Naraghi-Arani, P., Elsheikh, M., Carrillo, A.C., Williams, P.L., and Gardner, S.N. (2012). Skip the alignment: degenerate, multiplex primer and probe design using K-mer matching instead of alignments. PloS one. *7:*e34560.

Nelson, C.E., Kumar, D.M., Hemphill, E., Măndoiu, I.I., Duitama, J., and Khan, M. 2009. PrimerHunter: a primer design tool for PCR-based virus subtype identification. Nucleic Acids Res. 37:2483-2492.

Phillippy, A.M., Ayanbule, K., Edwards, N.J., and Salzberg, S.L. 2009. Insignia: a DNA signature search web server for diagnostic assay development. Nucleic Acids Res. 37: W229-W234.

Pritchard, L., Holden, N.J., Bielaszewska, M., Karch, H., and Toth, I.K. 2012. Alignment-Free Design of Highly Discriminatory Diagnostic Primer Sets for *Escherichia coli* O104:H4 Outbreak Strains. PloS one *7*, e34498.

Satya, R.V., Kumar, K., Zavaljevski, N., and Reifman, J. 2010. A high-throughput pipeline for the design of real-time PCR signatures. BMC bioinformatics *11*, 340.

Slezak, T., Kuczmarski, T., Ott, L., Torres, C., Medeiros, D., Smith, J., Truitt, B., Mulakken, N., Lam, M., and Vitalis, E. 2003. Comparative genomics tools applied to bioterrorism defence. Briefings in bioinformatics. 4:133-149.

Thomsen, M.C.F., Hasman, H., Westh, H., Kaya, H., and Lund, O. 2017. RUCS: rapid identification of PCR primers for unique core sequences. Bioinformatics (Oxford, England). 33: 3917-3921.

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., and Madden, T.L. 2012. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. BMC bioinformatics. 13:134.

CHAPTER III: IMPROVED DETECTION OF THE POTATO PATHOGEN *CLAVIBACTER MICHIGANENSIS* SUBSP. *SEPEDONICUS* USING DROPLET DIGITAL PCR

Synopsis

The Gram-positive bacterium *Clavibacter michiganensis* subsp. *sepedonicus* (Cms) causes bacterial ring rot in potato. There is zero tolerance for Cms in seed potato production, and the presence of a single infected plant may disqualify an entire farm from seed production for that year. Effective management of Cms is aided by accurate and sensitive molecular detection methods. Ambiguous test results have led to costly delays and occasionally to both financial and yield losses for farmers in subsequent growing seasons. In this study, we developed a droplet digital PCR (ddPCR) assay for accurate and sensitive detection of Cms. The assay was compared empirically to existing methods for detection including real-time PCR (RT-PCR), and ELISA, using field samples from a previous outbreak. The ddPCR assay improved detection capacity by 10-fold using serial dilution of genomic DNA compared to RT-PCR. The ddPCR assay is sensitive enough to consistently detect one infected potato tuber core among 800 uninfected tuber cores whereas RT-PCR is limited to one infected core in 400. As such, larger seed potato lots can be comprehensively sampled using ddPCR. Because of the improved sensitivity of ddPCR, Cms was found on potatoes sold for consumption in grocery stores in the course of assay development. This discovery highlights the improved detection capabilities of ddPCR and the need for expanded Cms monitoring.

1. Introduction

Clavibacter michiganensis subsp. *sepedonicus* (Davis, Gillaspies, Vidaver & Harris; Cms) is a Gram-positive bacterium that causes bacterial ring rot in potato. It was first described in Germany in 1906 (Appel 1906/1911) and first detected in the United States (US) in 1938 (Burkholder 1938). Since the early 1940s Cms has been treated as a zero-tolerance pathogen in seed potato production due to the large yield losses caused by the bacterium (De Boer and Slack 1984). Foliar symptoms of Cms differ across potato varieties and can be difficult to distinguish from natural plant senescence, making it challenging to diagnose. In addition, this slow-growing and difficult to isolate pathogen can survive for long periods on farm equipment and also can remain latent in seed potatoes for multiple cropping cycles (Franc 1999; Nelson 1980, 1982, 1984, 1985; Nelson and Kozub 1990). Therefore, seed producers and certifiers rely on molecular assays to screen for Cms in seed potato lots. Identification of Cms in a single plant or tuber of a seed lot results in rejection of the entire seed lot for planting, and triggers additional surveying of the farm to identify other seed lots that may carry Cms (Frost et al. 2013). A positive detection of Cms can result in up to 50% crop loss for the affected farmer and reduces seed potato supplies (Easton 1979).

Currently, Cms identification relies primarily on enzyme-linked immunosorbent assay (ELISA) and quantitative real-time PCR (RT-PCR) methods (De Boer et al. 2005; Gudmestad et al. 2009). Both methods use a threshold value to differentiate a negative result from positive result, however, values close to the threshold occur and these ambiguous results can lead to either false positives or false negatives. In addition, the method for calculating thresholds for RT-PCR and ELISA is not standardized across labs. Thresholds for RT-PCR assays are typically arbitrarily chosen based on experience (Gudmestad et al. 2009) and ELISA thresholds are either arbitrarily

chosen based on experience or one of multiple statistical methods may be used (De Boer and Hall 1996; Sutula et al. 1986). Because of these concerns and the stringent quarantine ramifications associated with positive Cms test results, seed potato certification agencies typically require Cms-positive test results from two distinct assays before designating a sample as positive. False negative results can be even more problematic as incorrectly diagnosed seed potatoes with Cms can spread the pathogen to additional seed lots and farms, resulting in potentially larger disease outbreaks in the subsequent cropping season.

Given the importance of Cms diagnoses in seed potato certification, a more sensitive and precise method is needed for improved identification of Cms in seed potatoes. A recent review of ddPCR use in pathogen detection (Kuypers and Jerome 2017) indicates that ddPCR could be well suited for improving the detection threshold of Cms in potato tissue. Recently, ddPCR assays for Plasmodiophora, as well as bacterial, and viral plant pathogens were reported to be equivalent or better than RT-PCR for pathogen detection from environmental samples (Dreo et al. 2014; Gossen et al. 2019; Liu et al. 2019; Selvaraj et al. 2018).

Proposed in the late 1990s (Vogelstein and Kinzler 1999), and developed in the early 2010s (Hindson et al. 2011), ddPCR relies on partitioning of a 20 µL PCR mixture into approximately 20,000 nanoliter-size droplets in which the reactions take place. This provides an improved statistical population to sample from compared to the typical single reactions of conventional PCR. The threshold cutoff values for ddPCR are easily and consistently determinable, and a false positive rate (FPR) can be empirically determined for each assay (Armbruster and Pry 2008; Zink et al. 2017). Due to the high level of partitioning in ddPCR, even a single copy of target DNA can be detected, giving a definitive positive or negative result for samples that are ambiguous when tested with RT-PCR (Tembrock et al. 2017). Since ddPCR occurs in such small volumes, it is more

resistant to the presence of PCR inhibiting compounds and is more sensitive to low template copy numbers than RT-PCR due to the lack of competition for reagents within each reaction (Dingle et al. 2013).

Assays for plant diseases have only recently been developed using ddPCR, with the first protocol published in 2014 (Dreo et al. 2014). Other examples of ddPCR applications using complex environmental samples include detection of a single insect species from hundreds of individuals from a congeneric species (Nathan et al. 2014; Zink et al. 2017; Zink et al. 2018], detection of invasive aquatics species, detection of genetically modified organisms in diverse plant samples (Giraldo et al. 2019), and detection of fecal contamination in water (Cao et al. 2015). Here we show that ddPCR is more sensitive than RT-PCR for Cms detection in field samples by simulating a seed lot with one inoculated potato tuber core combined with increasing numbers of uninfected cores. We evaluated ambiguous field samples with RT-PCR, ELISA, and ddPCR to determine where Cms was present. Our results also suggest that Cms was present in asymptomatic potatoes in local grocery stores.

2. Materials and Methods

2.1. Bacterial cultures

Bacterial strains used in this project are listed in Table 1. Bacterial isolates were streaked onto nutrient agar (NA) and grown at room temperature for one week prior to inoculation into tubers or for DNA extraction. Strains were stored at -80°C by suspending the cells in 20% vol: vol glycerol: water inside cryovials (Davis and Vidaver 2001).

2.2. Tuber core sample preparation

Tuber sample preparation followed that used for screening seed potato lots for Cms (De Boer et al. 2005). For the simulation tests a potato tuber was inoculated with Cms (SD-1) by

swiping a toothpick across a Cms culture growing on NA and piercing the potato. The tuber was then incubated at room temperature for seven days. An approximately 1 cm by 1 cm cylindrical core containing the inoculation site was taken from the potato tuber and mixed with 400 or 800 uninfected cores obtained from healthy tubers to test the sensitivity of the ddPCR assay. For negative controls, only uninfected cores were used. The groups of tuber cores were placed in sterile flasks, covered with sterile water, placed on a rotary shaker, and incubated at room temperature with low speed shaking overnight. DNA was then extracted from a 1 mL subsample of water as described in the section below.

To investigate colonization of Cms bacteria inside the potato tuber, we sampled Cms-inoculated potatoes at different sites. To do this we inoculated a potato tuber with Cms using a sterilized toothpick at the stem end of the tuber. The inoculated tubers were incubated at room temperature for 1 week. Two 1 cm by 1 cm cylindrical cores were taken from the tuber with sterile knives, one from the stem end and one from the bud end of the tuber. DNA was extracted from each individual core as described below to determine if Cms could be detected beyond the inoculation site.

Tuber samples were obtained from a commercial field that had been inadvertently planted with an infected seed lot. These samples were used to test the performance of the ddPCR assay with real-world field samples. Samples were obtained by taking 1 cm by 1 cm cylindrical cores from the stolon-end of individual asymptomatic tubers. Each core was placed into a sterile test tube, covered with sterile water, and incubated at room temperature on a rotary shaker at low speed overnight. One mL of the supernatant was removed and used for DNA extractions or ELISA as described above.

2.3. DNA extraction

DNA was extracted from 1 mL tuber core supernatant subsamples and from pure cultures with a FastDNA Spin Kit for Soil following the manufacturer's protocol (MP Biomedicals, USA). The quantity of DNA was determined for all samples with a NanoDrop 1000 spectrophotometer at 230 nm following the manufacturer's protocol (Software version 3.8.1; Thermo Fisher Scientific, Waltham, Massachusetts, USA). Purified genomic DNA was stored at -20°C until use.

2.4. ELISA and RT-PCR assays

ELISA for Cms detection was performed using the double antibody sandwich kit (DAS-ELISA) available from Agdia, Inc. (Elkhart, Indiana, USA). Each ELISA well was loaded with 100 µl of bacterial suspension from the soaked tuber cores and the assays were processed following the manufacturer's recommendations. ELISA tests were evaluated using an Awareness Technologies Stat Fax 2100 Microplate Reader (GMI, Ramsey, Minnesota, USA) at a wavelength of 405 nm.

For the RT-PCR assays, we used primer pair CelA (Gudmestad et al. 2009) or primer pair Cms72 in simplex assays (Mills et al. 1997) and detected the amplified DNA with a probe (Table 2). We chose to adapt the Cms72 assay for RT-PCR because the primer sets have been successfully used previously for Cms detection whereas primer set Cms85 frequently amplifies non-Cms DNA from potato tuber samples and primer set Cms50 is less sensitive (Charkowski, personal observations). The RT-PCR assay was performed for both CelA and Cms72 assay in a final volume of 25 µL, containing 10 µL of Prime Time Gene Expression Master Mix (IDT Inc., San Jose, California, USA), 0.75 µM of each primer, 0.16 µM of probe, and 2 µL of 100 ng/µL of genomic DNA. The thermocycling protocol for RT-PCR was an initial incubation of the mix at 95°C for 10 min followed by 40 cycles at 95°C for 30 s, 60°C for 45 s, and 72°C for 30 s with data

capture at the end of each 72°C cycle with lid temperature of 105°C in an ABL-700 real-time PCR system (Applied Biosystems, Foster City, California, USA).

2.5. ddPCR assays

The CelA primers and probe previously described were compared to the Cms72 primers for the ddPCR assay. To adapt the Cms72 assay for ddPCR, we manually designed a probe between the Cms 72 forward and Cms 72 reverse primers (Table 2). The RT-PCR assay was optimized for ddPCR by fragmenting the input DNA, sequentially decreasing the amount of primers and probes in each reaction, and altering the thermal cycling protocol to be consistent with the chemistry and the thermal properties of the aqueous droplets. After extraction, all DNA samples used for ddPCR were run through QIAshredder columns (Qiagen, Valencia, California, USA) to fragment the DNA for efficient packaging into droplets. A master mix was then made using 10 µL Supermix for probes (no dUTP) (Bio-Rad Laboratories Inc., Hercules, California, USA), 500 nM forward and reverse primers, 250 nM probe (Table 2), and enough water to bring the final volume to 19 µl/reaction. The master mix was then vortexed for 10 sec, spun down briefly, vortexed for 10 sec again, and spun down a final time before it was aliquoted into 0.2 mL tubes and 1 µL of shredded sample DNA was added to each reaction. The tubes were centrifuged briefly, and the reaction mixture was added to the middle wells of a disposable DG8 cartridge for the QX100/QX200 Droplet Generator (Bio-Rad Laboratories Inc.). This was followed by the addition of 70 µL of droplet generation oil for probes (Bio-Rad Laboratories Inc.) to the bottom wells of the cartridge, which was sealed with a disposable DG8 Gasket and placed in the QX200 Droplet Generator System (Bio-Rad Laboratories Inc.) and droplet generation was carried out. The droplets were then transferred from the top wells of the cartridge to an Eppendorf semi-skirted 96-well plate (Eppendorf AG, Hamburg, Germany) using an Eppendorf Xplorer Plus 5-100 µL automatic

pipettor (Eppendorf AG) set to the lowest draw and expel speeds to maximize droplet recovery. The plate was sealed using Bio-Rad Pierceable Foil Heat Seal (Bio-Rad Laboratories Inc.) in a PX1 PCR plate sealer (Bio-Rad Laboratories Inc.). Thermal cycling was carried out in a Bio-Rad C1000 thermal cycler with a deep-well reaction module (Bio-Rad Laboratories Inc.) following the protocol: (1) 95°C for 10 min, (2) 95°C for 30 sec, (3) 58°C for 45 sec, (4) 72°C for 30 sec, (5) repeat steps 2-4 39 times, (6) 98°C for 10 min, followed by an infinite hold at 10°C, lid temperature of 105°C throughout. A ramp rate of 2°C/sec was used between all steps of the thermal cycle program to ensure consistent heating and cooling of all droplets.

After thermal cycling, the plate was placed in the block of a Bio-Rad QX200 Droplet Reader (Bio-Rad Laboratories Inc.) and droplets were read at a rate of 32 wells/ hour. Data were analyzed using QuantaSoft version 1.7.4 and QuantaSoft Analysis Pro version 1.0 (Bio-Rad Laboratories Inc.). The threshold for positive droplets was determined using the Javascript "definetherain" (definetherain.org.uk; 2014) based on positive control data for each ddPCR run. Purified Cms DNA diluted 1:1000 in water was used as a positive control for all runs. Using the set threshold, Poisson statistics were recalculated by QuantaSoft.

2.6. False positive rate and limit of detection for ddPCR.

In order to determine a cutoff for the number of false positives for each primer set, both were used to run 65 replicates of DNA extraction from uninfected potato cores as negative controls. Purified, diluted Cms DNA was run as a positive control for the fluorescence amplitude cut off below which droplets are negative and above which they are positive. Using this data, the False Positive Rate (FPR) for the CelA and Cms72 assays were determined by combining the total number of positive droplets in the negative control wells divided by the total number of wells run. This data was used to determine FPR call thresholds, which were then used to define the limit of

detection (LoD) for each ddPCR assay using look-up tables. The look-up tables were provided by Bio-Rad as modified from Armbruster and Pry (Armbruster and Pry 2008).

3. Results

3.1 ddPCR sensitivity in detection of purified Cms DNA

The CelA primers and probe and the Cms72 primers and probe (Table 2) were first tested using RT-PCR with 100 ng/µL of Cms DNA. The crossing threshold (C_t) value of 35 cycles was used in all experiments to determine presence of Cms (Gudmestad et al. 2009) (Table 3). The ddPCR assay was optimized as reflected in the materials and methods with the Cms strain SD-1 (Table 1).

The sensitivity of the ddPCR assay for the target loci was tested with DNA isolated from pure cultures. The DNA was diluted to an initial concentration of 10 ng/µL and then further diluted to $1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$, $1:10^6$, $1:10^7$, and $1:10^8$ in sterile water. Cms was detectable in all dilutions down to $1:10^6$, with a single droplet present for each primer set at $1:10^7$ (Fig 1A, B). The CelA and Cms72 primer sets have been successfully used by the potato industry for many years for Cms detection, and their specificity has already been thoroughly tested. We further confirmed this by testing for off-target amplification with each primer/probe set with related *C. michiganensis* species, including three strains of *C. michiganensis* subsp. *michiganensis* (Cmm), which causes tomato canker, and three strains of *C. michiganensis* subsp. *nebraskensis*, which causes Goss's wilt of maize (Cmn; Table 2). The Cms72 primer set had no off-target amplification with the related subspecies, but there was some off-target amplification by CelA for all four strains of Cmn tested (Fig 1A, B).

3.2. Use of ddPCR to detect Cms in low-titer field samples

DNA extracted from individual asymptomatic tubers from a Cms outbreak on a commercial farm were tested with ddPCR, RT-PCR, and ELISA. Individual tubers were used because we wished to obtain a range of Cms concentrations from field samples. Of the 20 samples tested, 11 samples were consistently positive, and three samples were consistently negative with ELISA, RT-PCR and ddPCR assays. Unlike with RT-PCR assays, both ddPCR assays gave identical results with all 20 of these field samples. Three of the assays were positive by ELISA, but not with any of the PCR assays. Two of the samples gave ambiguous results with the RT-PCR assays and were rated as negative by ELISA. Of these, one was positive in both ddPCR assays and the other was negative in both ddPCR assays. (Table 3).

3.3. Cms is detectable in bulk samples of up to 800 potato cores

We inoculated tubers with Cms to test the efficacy of the RT-PCR and ddPCR assays in testing by bulk sampling. In our initial experiment, two cores were taken from each tuber, one from the stem end and one from the bud end of the tuber (Fig 2A, B). After DNA extraction, both core samples were used in a ddPCR assay for Cms detection. High levels of Cms were detected at both tuber sample sites using both primer sets (Fig 2A, B).

European Community Directive 93/85/EEC recommends that commercial testing for Cms should carried out in lots of 200 potatoes at a time by PCR. To determine if a greater number of tubers can be tested in each sample, a single infected potato core was included with samples of 400, and 800 uninfected potato cores. Using ddPCR, Cms was detectable in bulk tests using 400 and 800 cores for both primer sets (Fig 2C, D). A lot consisting of 200 uninfected potato cores was used as a negative control to determine whether the potatoes used were free of Cms contamination

prior to inoculation and to ensure that non-target DNA did not interfere with the assay (Fig 2C, D).

3.4. Detection of Cms in commercial potatoes using ddPCR

We tested potato tubers from a local grocery store during the course of assay development. Our results revealed that the initial two sets of samples had detectable levels of Cms using CelA and Cms72 assays (Table 4). We surveyed additional samples of commercial potatoes of three different potato varieties from two states to determine whether Cms contamination is widespread. We detected Cms in one of six samples with the CelA assay, and in all six samples with the Cms72 assay (Table 4).

3.5. False positive rate

The false positive rate (FPR) for the CelA assay was determined empirically to be 0.11 false positive events per well. At this rate, the threshold to call the well positive at a 99% confidence interval (CI) is two positive droplets and the limit of detection is seven copies per well. For the Cms72 assay, the FPR was determined to be zero. From this a positive call threshold at a 99% CI is one positive droplet per well a limit of detection of five copies per well (Fig S1).

4. Discussion

Bacterial ring rot in potato is caused by Cms, which is treated as a zero-tolerance pathogen throughout North America and Europe. Control of Cms is difficult due to its ability to remain latent inside host tissues for long periods of time (Franc 1999; Nelson 1982). Despite attempts at eradication, it is still detected in seed potatoes and Cms is still the cause of occasional costly outbreaks. Limitations with current Cms detection assays have likely contributed to the spread of Cms through planting of infected seed potatoes that were falsely determined to be uninfected. The ddPCR assay developed here is at least 10-fold more sensitive than RT-PCR with DNA from pure

Cms cultures. The ddPCR assay can also detect Cms in an 800-tuber composite sample. In addition to improved sensitivity, ddPCR assays do not require an external calibration curve to define the threshold to use for determining whether the pathogen is present (Hindson et al. 2011), unlike ELISA or RT-PCR. We took advantage of this to determine whether Cms was present in asymptomatic field samples that had ambiguous or conflicting results with RT-PCR and ELISA assays.

We tested two previously developed primer sets Cms72 and CelA, both of which are widely used for Cms detection in potato. The CelA primer set amplifies a region upstream of Cms *celA*, which is plasmid-encoded virulence gene (Gudmestad et al. 2009; Holtsmark et al. 2007; Laine et al. 2000), and Cms72 amplifies a portion of a gene of unknown function (Bentley et al. 2008; Mills et al. 1997). We found that the Cms72 primer set is better suited for ddPCR. The CelA assay was less sensitive than the Cms72 assay, it amplified non-target DNA from the closely related *C. michiganensis* subsp. *nebraskensis*, and it had reduced separation between positive and negative droplets and a greater number of rain droplets. After observing these results, we used BLASTN to reevaluate the CelA primers and found that the CelA primers and probe contain repetitive sequences of 9 to 15 nucleotides (Altschul et al. 1990). These sequences are found throughout the *C. michiganenesis* genome and PCR-mediated recombination between these repetitive sites could result in off-target amplification (Potapov and Ong 2017). In contrast, the Cms72 primers and probe do not contain repetitive sequences. The repetitive sequences in the CelA primers and probe could explain all of the deficiencies in the CelA assay compared to the Cms72 assay. As a whole, our results demonstrate that assays developed for RT-PCR, such as the CelA assay, may not be suitable for ddPCR and that the Cms72 primer/probe set is more appropriate for ddPCR detection of Cms.

The inherent resistance of ddPCR to PCR inhibitors (Dingle et al. 2013; Quan et al. 2018) and the ease and consistency with which threshold cutoff values and false positive rate (FPR) can be empirically determined (Armbruster and Pry 2008; Zink et al. 2017) make ddPCR an excellent alternative to ELISA and RT-PCR. Because of these attributes, ddPCR assays for Cms in large bulk samples will have greater throughput and precision than ELISA and RT-PCR assays. We tested this with samples containing tuber cores from up to 800 potatoes to mimic pre-planting seed potato testing and with field samples that had previously given ambiguous results with RT-PCR and ELISA.

The number of individual tuber samples taken from a seed potato lot determines the confidence interval for finding the pathogen (Clayton and Slack 1988), but testing costs impose a limit on how many tubers are tested in practice. The most common sample size for seed tuber testing in North America is 400 tubers per seed lot, which are generally tested in two pools of 200 tubers each, and this sample size is primarily chosen to balance assay sensitivity, disease risk, and assay costs. If the lot is negative for the pathogen, the inference is that the pathogen is present at less than 1% incidence with a 95% confidence level. For the zero-tolerance pathogen Cms, growers may test a larger number of tubers per seed lot, typically 1200 per lot, particularly if there has been a recent outbreak in the region. The larger sample size used is, again, a balance of sensitivity, risk, and cost.

In our initial experimental design, we had planned to determine the greatest number of cores that could be tested in a single bulk sample. However, obtaining tubers from seed or commercial farms for testing Cms assays is risky for the farmer because if the pathogen is found, it can lead to additional testing and large financial losses for seed potato farmers. Therefore, we decided to use tubers from a local grocery store so if Cms was found, it could not easily be traced

back to a specific farm. These tubers may have become infested with Cms during packaging and shipping. Unfortunately, a low level of Cms was detected in all of these grocery store samples, making them unsuitable for further assay development. We were able to obtain a limited number of tubers from the Colorado State University Potato Research Station, and these tubers tested negative for Cms in all of our assays. As such we were only able to test bulk samples containing 800 tuber cores.

We found that our ddPCR assay could unambiguously detect 1 infected tuber core mixed in a bulk sample with 800 uninfected tuber cores. It is likely that a bulk sample larger than 800 tuber cores could be effectively run for the detection of low levels of Cms using the ddPCR assay described in this paper. Larger bulk samples were not tested due to the difficulty in obtaining enough field-grown tubers from a location where the risk of finding the pathogen was acceptable to the farmer. Regardless, the ability to test 800-tuber bulk samples for Cms detection is an improvement over current protocol.

We collected asymptomatic tubers from a Cms outbreak to attempt to obtain field samples that had varying levels of the pathogen. Of these 20 samples, six had ambiguous or conflicting results with ELISA and RT-PCR, which are assays currently in use for Cms detection. Our ddPCR assay only amplified DNA from one of these six ambiguous samples, suggesting that the other five ambiguous samples were truly negative for Cms (table 3).

Notably, the CelA primer set, which had a high amount of rain in the ddPCR assay and contained repetitive tRNA sequence motifs also amplified a low level of DNA from five of the six ambiguous samples. This suggests that the problems observed in the ddPCR assay with the CelA primer set also plague the RT-PCR assay. As described by Przewodowski and Prezewodowska (Przewodowski and Przewodowska 2017) non-specific reactions (false positives) frequently occur

with current ELISA methods. Limited ELISA specificity is a reasonable explanation for the three ambiguous samples that were positive by ELISA, but negative with the RT-PCR and ddPCR assays. We expected ELISA to be less sensitive than the PCR assays but observed that ELISA gave positive results in all but one sample that tested positive for Cms with RT-PCR and ddPCR. However, because ELISA also gave false positive results with three tubers from this sample set, we do not know if the ELISA results from samples with low pathogen levels are true positives. These experiments with field samples spanning a range of Cms concentrations demonstrates the challenges of Cms diagnosis, the limitations of the current ELISA and RT-PCR assays, and the utility of the ddPCR assay as a detection tool.

During our experiments, we initially used grocery store samples to develop the detection assays but found a low level of Cms in all of the samples tested. The Cms found in these samples may have infested the potato tubers on the farm where they were grown or during shipping, washing, or packaging. There have been multiple outbreaks in the past decade in the western half of North America, so the presence of Cms in commercial potatoes is not surprising. We were unable to confirm the presence of Cms in these samples with a separate assay because the concentrations of Cms detected were below the limit of detection for RT-PCR. Confirmation by isolation of the bacteria also was not possible. There is no selective medium available for Cms and on general or semi-selective medium, Cms is overgrown by other, more quickly growing environmental bacteria. Despite the inability to confirm the results of ddPCR assays with very low levels of pathogen, this method may be useful in determining where Cms may be present on farms that are already known to be part of a Cms outbreak.

Overall, our results demonstrate that ddPCR offers a reliable, robust alternative to RT-PCR and ELISA for Cms testing in seed potatoes and, because of the increased sensitivity, ddPCR might

also be useful for monitoring Cms in farm ecosystems. The cost associated with ddPCR is comparable with RT-PCR. However, as ddPCR has a greater sensitivity and an improved method for threshold determination, less time may be needed to evaluate samples that would have had ambiguous test results with ELISA or RT-PCR. The greater sensitivity of ddPCR allows for bulking a greater number of tubers into a single sample, which further offsets the cost of ddPCR. Our results support the use of ddPCR technology with the Cms72 primer and probe set as a primary screening tool when indexing certified seed lots for the presence of Cms.

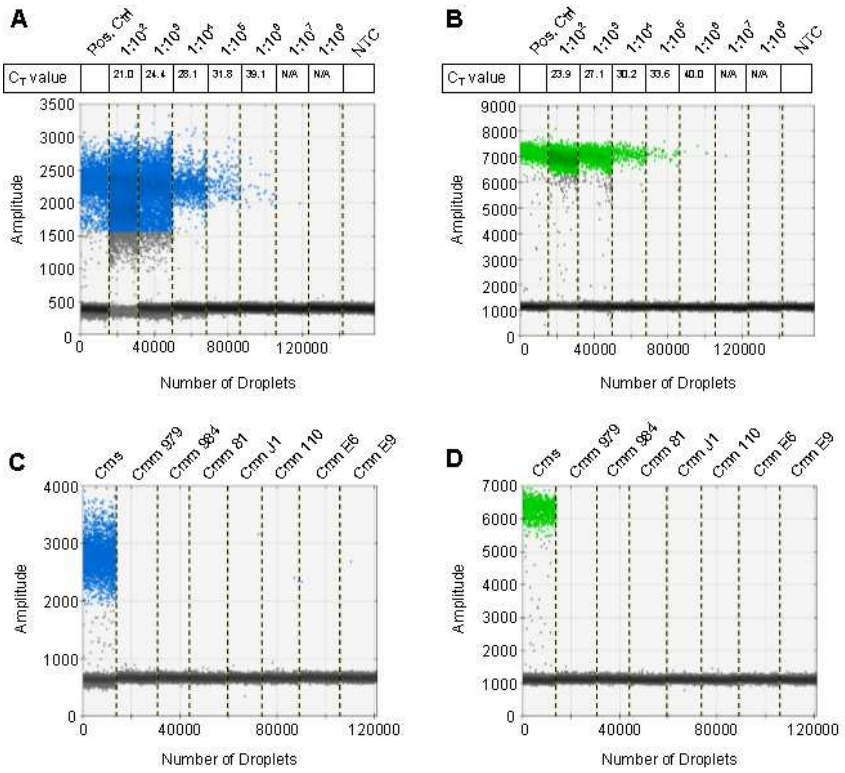


Figure 3.1. The primer/probe sets for Cms72 and CelA are sensitive and specific. Primer/probe sets for detection of A, CelA and B, Cms72 from Cms were used to detect serial dilutions of purified Cms DNA, and to check for cross reaction with *C. michiganensis* subsp. *michiganensis* (Cmm) and, C. *Clavibacter michiganensis* subsp. *nebraskensis* (Cmn) using CelA C, and Cms72 D. Thresholds were defined using definetherain.

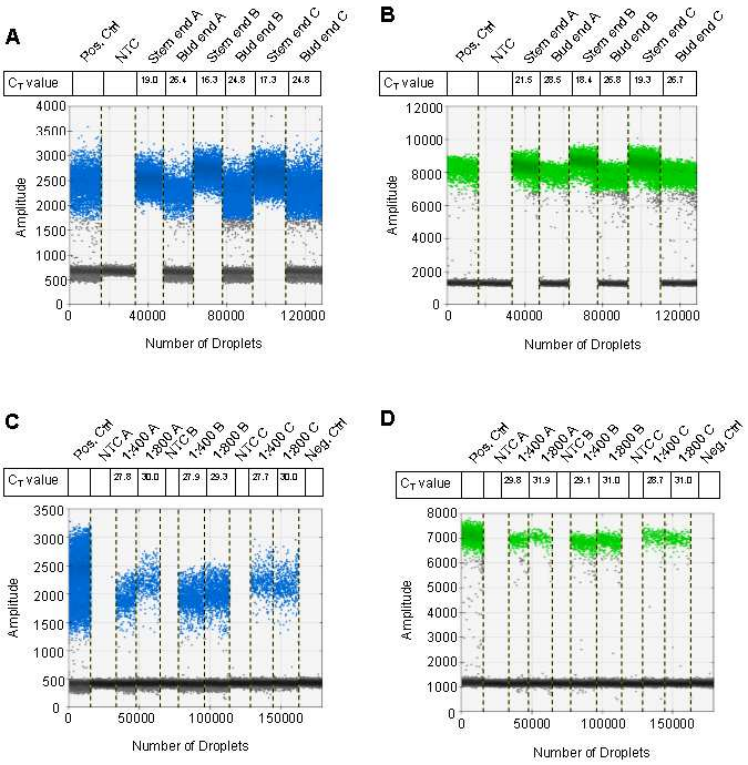


Figure 3.2. Cms is detectable in individual and bulk samples of potatoes. Primer/probe sets for A, CelA and B, Cms72 were run on individual infected potatoes which were each sampled at the stem end and bud end on the tuber. Bulk samples of 400 or 800 tubers were tested with C, CelA and D, Cms 72.

Table 3.1. Bacterial isolates used in this study

Species	**Geographic origin**	**Source[a]**	**Number of isolates**
Clavibacter michiganensis subsp. *sepedonicus*	South Dakota, Wisconsin	Gudmestad, Clarke	6
Clavibacter michiganensis subsp. *michiganensis*	New York	Smart	3
Clavibacter michiganensis subsp. *nebraskensis*	Colorado	Ned	3

[a] Isolates were provided by following sources. Gudmestad: SD-1, INM, AS-1, OFF; Clarke: C.sep.1 (tiny, non-mucoidal), C.sep.1 (medium-small); Smart: 0417(Cmm), 0420B(Cmm), 0445A; Ned: 428(E6-E7), B22(110), B23(J1).

Table 3.2. Primers and probes used in this study

Primer/probe	Sequence (5'-3')	Citation
CelA-F	TCTCTCAGTCATTGTAAGATGAT	Gudmestad et al. 2009
CelA-R	TTCGACCGCTCTCAAA	
CelA probe	[DHEX] TTCGGGCTTCAGGAGTGCGTGT [DBH2]	
Cms85 F	AAGATCAGAAGCGACCCGC	(Mills et al. 1997)
Cms85 R	GCTGGATTTGGCTGTGCGA	
Cms50 F	GAGCGCGATAGAAGAGGAACTC	
Cms50 R	TTTTCTTGTCGTTGCTCAGGA	
Cms72 F	GTTCGAGTTGATAGCAATCC	
Cms72 R	GGTGATCGTGAATCCGAGACAC	
Cms72 probe	[HEX] ATCGCAGACGCAGGTTTCAATCCG[ZEN]	This Study

Table 3.3. Comparison Cms detection methods with asymptomatic tubers

	ddPCR		RT-PCR			
Sample	CelA	Cms72	CelA C_t[a]	Cms72 C_t	ELISA	Conclusion
1	+	+	20.13	21.93	+	+
2	+	+	20.60	22.83	+	+
3	+	+	20.74	22.43	+	+
4	+	+	22.39	25.04	+	+
5	+	+	23.14	25.30	+	+
6	+	+	24.04	25.49	+	+
7	+	+	27.02	29.16	+	+
8	+	+	28.90	30.46	+	+
9	+	+	32.01	33.16	+	+
10	+	+	32.18	33.68	+	+
11	+	+	33.00	34.00	+	+
12	+	+	34.92	36.08	-	ambiguous
13	-	-	35.41	n/a	-	ambiguous
14	-	-	37.66	n/a	+	ambiguous
15	-	-	39.05	n/a	-	ambiguous
16	-	-	39.10	n/a	+	ambiguous
17	-	-	n/a	n/a	+	ambiguous
18	-	-	n/a	n/a	-	-
19	-	-	n/a	n/a	-	-
20	-	-	n/a	n/a	-	-

[a]C_t <35 is considered as positive for Cms using CelA and Cms72 primers (Gusmestad et al. 2009).

Table 3.4. Grocery store samples

Sample	CelA Positive Droplets[a]	Cms72 Positive Droplets
Grocery Store 1 (3 reactions)	18	4
Grocery Store 2 (14 reactions)	5	26
Colorado 1	0	3
Colorado 2	0	9
Colorado 3	0	6
Idaho 1	0	2
Idaho 2	0	5
Idaho 3	0	1

[a] Positive droplet represent the total number of positives obtained for each sample.

REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403-410.

Appel, O. 1906/1911. Die bakterienringkrankheit der kartoffel. Kaiserliche biologische Anstalt für Landund Forstwirtschaft Flugblatt 36.

Armbruster, D. A., and Pry, T. 2008. Limit of blank, limit of detection and limit of quantitation. Clinical Biochemist Reviews Suppl 1:S49-52.

Bentley, S. D., Corton, C., Brown, S. E., Barron, A., Clark, L., Doggett, J., Harris, B., Ormond, D., Quail, M. A., May, G., Francis, D., Knudson, D., Parkhill, J., and Ishimaru, C. A. 2008. Genome of the actinomycete plant pathogen *Clavibacter michiganensis* subsp. *sepedonicus* suggests recent niche adaptation. Journal of Bacteriology 190:2150-2160.

Burkholder, W. H. 1938. The occurrence in the United States of tuber ring rot and wilt of the potato. Amer. Potato J. 15:243-245.

Cao, Y., Raith, M. R., and Griffith, J. F. 2015. Droplet digital PCR for simultaneous quantification of general and human-associated fecal indicators for water quality assessment. Water Research 70.

Clayton, M. K., and Slack, S. A. 1988. Sample-Size Determination in Zero Tolerance Circumstances and the Implications of Stepwise Sampling - Bacterial Ring Rot as a Special Case. American Potato Journal 65:711-723.

Davis, M. J., and Vidaver, A. K. 2001. Coryneform Plant Pathogens. Pages 281-234 in: Laboratory Guide for Identification of Plant Pathogenic Bacteria. J. B. J. N. W. Schaad, and W. Chun, ed. APS Press, St. Paul, MN.

De Boer, S. H., and Slack, S. A. 1984. Current status and prospects for detecting and controling bacterial ring rot of potatoes in North America. Plant Disease:821-844.

De Boer, S. H., and Hall, J. W. 1996. The probability of detecting *Clavibacter michiganensis* subsp. *sepedonicus* by indexing seed potato lots with serological tests. J. Phytopathol. 144:459-463.

De Boer, S. H., Charkowski, A. O., Zink, R. T., Martínez-Soriano, J. P., and Flores-Olivas, A. 2005. Procedure for detection and identification of *Clavibacter michiganensis* subsp. *sepedonicus* (Spiekermann and Kotthoff) David, Gillaspie, Vidaver and Harris, in potato (*Solanum tuberosum* L.) tubers. . Revista Mexicana de Fitopatología 23:329-334.

Dingle, T. C., Sedlak, R. H., Cook, L., and Jerome, K. R. 2013. Tolerance of droplet-digital PCR versus real-time quantitative PCR to inhibitory substances. Clinical Chemistry 59:1670-1672.

Dreo, T., Pirc, M., Ramšak, Ž., Pavšič, J., Milavec, M., Zel, J., and Gruden, K. 2014. Optimising droplet digital PCR analysis approaches for detection and quantification of bacteria: a case study of fire blight and potato brown rot. Analytical and Bioanalytical Chemistry 406:6513-6528.

Easton, G. D. 1979. The biology and epidemiology of potato ring rot. American Potato Journal 56:459-460.

Franc, G. D. 1999. Persistence and latency of *Clavibacter michiganensis* subsp. *sepedonicus* in field-grown seed potatoes. Plant Dis 83:247-250.

Frost, K. E., Groves, R. L., and Charkowski, A. O. 2013. Integrated control of potato pathogens through seed potato certification and provision of clean seed potatoes. Plant Dis. 97:1268-1280.

Giraldo, P. A., Cogan, N. O. I., Spangenberg, G. C., Smith, K. F., and Shinozuka, H. 2019. Development and application of droplet digital PCR tools for the detection of transgenes in

pastures and pasture-based products. Frontiers in Plant Science:https://doi.org/10.3389/fpls.2018.01923.

Gossen, B. D., Al-Daoud, F., Dumonceaux, T., Dalton, J. A., Peng, G., Pageau, D., and McDonald, M. R. 2019. Comparison of techniques for estimation of resting spores of *Plasmodiophora brassicae* in soil. Plant Pathology 68:954-961.

Gudmestad, N. C., Mallik, I., Pasche, J. S., Anderson, N. R., and Kinzer, K. 2009. A real-time PCR assay for the detection of *Clavibacter michiganensis* subsp. *sepedonicus* based on the cellulase A gene sequence. Plant Dis. 93:649-659.

Hindson, B. J., Ness, K. D., Masquelier, D. A., Belgrader, P., Heredia, N. J., Makarewicz, A. J., Bright, I. J., Lucero, M. Y., Hiddessen, A. L., Legler, T. C., Kitano, T. K., Hodel, M. R., Petersen, J. F., Wyatt, P. W., Steenblock, E. R., Shah, P. H., Bousse, L. J., Troup, C. B., Mellen, J. C., Wittmann, D. K., Erndt, N. G., Cauley, T. H., Koehler, R. T., So, A. P., Dube, S., Rose, K. A., Montesclaros, L., Wang, S., Stumbo, D. P., Hodges, S. P., Romine, S., Milanovich, F. P., White, H. E., Regan, J. F., Karlin-Neumann, G. A., Hindson, C. M., Saxonov, S., and Colston, B. W. 2011. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Analytical Chemistry 83:8604-8610.

Holtsmark, I., Takle, G. W., and Brurberg, M. B. 2007. Expression of putative virulence factors in the potato pathogen *Clavibacter michiganensis* subsp. *sepedonicus* during infection. Archives of Microbiology 189:131-139.

Kuypers, J., and Jerome, K. R. 2017. Applications of digital PCR for clinical microbiology. Journal of Clinical Microbiology 55:1621-1628.

Laine, M. J., Haapalainen, M., Wahlroos, T., Kankare, K., Nissinen, R., Kassuwi, S., and Metzler, M. C. 2000. The cellulase encoded by the native plasmid of Clavibacter michiganensis subsp.

sepedonicus plays a role in virulence and contains an expansin-like domain. Physiological and Molecular Plant Pathology 57:221-233.

Liu, Y. J., Wang, Y. L., Wang, Q., Zhang, Y. H., Shen, W. X., Li, R. H., Cao, M. J., Chen, L., Li, X., Zhou, C., and Zhou, Y. 2019. Development of a sensitive and reliable reverse transcription droplet digital PCR assay for the detection of citrus yellow vein clearing virus. Archives of Virology 164:691-697.

Mills, D., Russell, B. W., and Hanus, J. W. 1997. Specific detection of *Clavibacter michiganensis* subsp. *sepedonicus* by amplification of three unique DNA sequences isolated by subtraction hybridization. Phytopathology 87:853-861.

Nathan, L. M., Simmons, M., Wegleitner, B. J., Jerde, C. L., and Mahon, A. R. 2014. Quantifying Environmental DNA Signals for Aquatic Invasive Species Across Multiple Detection Platforms. Environmental Science & Technology 48:12800-12806.

Nelson, G. A. 1980. Long-term survival of *Corynebacterium sepedonicum* on contaminated surfaces and in infected potato stems. Am. Potato J. 57:595-600.

Nelson, G. A. 1982. Corynebacterium sepedonicum in potato: effect of inoculum concentration on ring rot symptoms and latent infection. Can. J. Plant Pathol. 4:129-133.

Nelson, G. A. 1984. Survival of Corynebacterium-Sepedonicum in Infected Potato Stems and on Contaminated Surfaces Held at Freezing Temperatures. American Potato Journal 61:532-532.

Nelson, G. A. 1985. Survival of Corynebacterium-Sepedonicum in Potato Stems and on Surfaces Held at Freezing and above-Freezing Temperatures. American Potato Journal 62:23-28.

Nelson, G. A., and Kozub, G. C. 1990. Survival of Corynebacterium-Sepedonicum at Freezing and at Wide Fluctuations between Freezing and above-Freezing Temperatures. American Potato Journal 67:625-631.

Potapov, V., and Ong, J. L. 2017. Examining sources of error in PCR by single-molecule sequencing. PLoS One 12:e0169774.

Przewodowski, W., and Przewodowska, A. 2017. Development of a sensitive and specific polyclonal antibody for serological detection of *Clavibacter michiganensis* subsp. *sepedonicus*. PLoS One 12:e0169785.

Quan, P.-L., Sauzade, M., and Brouzes, E. 2018. dPCR: A Technology Review. Sensors (Basel) 18:1271.

Selvaraj, V., Maheshwari, Y., Hajeri, S., Chen, J. C., McCollum, T. G., and Yokomi, R. 2018. Development of a duplex droplet digital PCR assay for absolute quantitative detection of "Candidatus Liberibacter asiaticus". PLoS One 13:e0197184.

Sutula, C. L., Gillett, J. M., Morrissey, S. M., and Ramsdell, D. C. 1986. Interpreting ELISA data and establishing the positive-negative threshold. Plant Disease 70:722-726.

Tembrock, L. R., Farris, R. E., Ledezma, L., N. B. Barr, N. B., and Gilligan, T. M. 2017. A real-time PCR assay for the separation of *Autographa gamma* (Noctuidae: Plusiinae) from morphologically similar species in North America. Journal of Economic Entomology 110:2609-2617.

Vogelstein, B., and Kinzler, K. W. 1999. Digital PCR. Proceedings of the National Academy of Science USA 96:9236-9241.

Zink, F. A., Tembrock, L. R., Timm, A. E., and Gilligan, T. M. 2018. A ddPCR assay for identification of *Autographa gamma* (Noctuidae: Plusiinae) in bulk trap samples Journal of Economic Entomology 111:1490-1495.

Zink, F. A., Tembrock, L. R., Timm, A. E., Farris, R. E., Perera, O. P., and Gilligan, T. M. 2017. A droplet digital PCR (ddPCR) assay to detect *Helicoverpa armigera* (Lepidoptera: Noctuidae) in bulk trap samples. PLoS One 12:e0178704.

CHAPTER IV: FROM METABOLOMICS TO FUNCTIONAL PHENOTYPES: REVEALING METABOLIC FEATURES OF DIPLOID POTATO SPECIES (*SOLANUM CHACOENSE*) IN RESPONSE TO BLACKLEG DISEASE

Synopsis

Some lines of the wild diploid potato *Solanum chacoense* are resistant or tolerant to blackleg and soft rot caused by *Dickeya*. To identify candidate metabolites that might contribute to resistance, we first screened 154 accessions of *S. chacoense* with a virulence assay to identify resistant and susceptible *S. chacoense* lines. We then used bio-chemical assays and non-targeted liquid chromatography mass spectrometry (LC-MS) to further characterize two resistant and two susceptible lines. Stem extracts from these selected accessions were tested for their effects on exoenzyme activity, cell morphology, AHL production, and bacterial motility of *Dickeya dianthicola* (ME23). *D. dianthicola* grown in stem extract from resistant lines had reduced protease (Prt), cellulase (Cel), pectate lyase (Pel) activity and reduced AHL synthesis compared to cells grown in the stem extracts from susceptible lines. However, stem extracts caused no difference in bacterial motility. A metabolic profile was created for resistant and susceptible lines, with and without *D. dianthicola* infection, using reverse phase LCMS. Analysis of metabolomic data showed clear distinction in the metabolites and their abundance between different *S. chacoense* lines. We found slight to very little shift in metabolic abundance in *S. chacoense* lines when infected with *D. dianthicola* at different timepoints. Detailed analysis and annotation identified phenolic acids, alkaloids, terpenes and fatty acids as potential contributors to resistance, potentially through interference with bacterial quorum sensing.

1. Introduction

Potatoes are grown worldwide and are the fourth most important staple food source after wheat, rice, and corn (De Boer and Isael 2004). In the past few years, US potato production has increased in value and, in 2019, reached approximately $4 billion. However, this important crop is facing constant threats from numerous bacterial, viral, and fungal pathogens, many of which cause significant annual losses worldwide (Essarts et al. 2016; Zeng et al. 2019; Nolte et al. 2004; Frost et al. 2013).

Blackleg disease of potato is one of the most important potato diseases worldwide. This disease is caused by soft rot Pectobacteriaceae (SRP), including *Dickeya* and *Pectobacterium* (Charkowski 2018; Pérombelon 2002). Recently, *Dickeya dianthicola* caused a multi-year outbreak in potato, raising interest in this pathogen (Karim et al. 2019; Nasaruddin et al. 2019; Hao et al. 2016, Ma et al. 2018; Jiang et al. 2016; Johnson 2016). *D. dianthicola* infects potato plants through natural openings or wounds and disease progression is affected by moisture, temperature, and potato variety. The pathogen spreads through infected seed potatoes, irrigation water, insects, equipment and tools (Reverchon and Nasser 2013). Once inside the plant, it secretes plant cell wall degrading enzymes (PCWDE), which macerate the plant cell wall and allow the bacteria to acquire nutrients from the host (Czajkowski et al. 2011, Charkowski et al. 2012, Panda et al. 2016; Pérombelon 2002). The bacterial maceration of plant cell walls is the cause of the wilt and decay symptoms characteristic of blackleg disease. Blackleg bacteria can also cause soft rot on potato tuber during any stage of production cycle (Charkowski 2018).

Dickeya and *Pectobacterium* species regulate numerous virulence genes with an N-acyl homoserine lactone (AHL) mediated quorum sensing (QS) system (Barnard et al. 2007; Liu et al. 2008). Virulence genes are also regulated by plant phenolics, plant cell wall fragments, oxygen

level, and temperature (Charkowski et al. 2012). Because of the key role that QS plays in virulence gene regulation in these pathogens, disruption of this system is a major target for development of resistant plants. Transgenic plants capable of degrading the AHL QS signal are resistant to *Pectobacterium*, but they are not accepted for use by consumers (Ragunath et al. 2012; Wang et al. 2010).

Plant resistance is the most desirable form of disease resistance, but it is an elusive target for blackleg in commercial potato. Potato plants produce protective molecules in both tubers and stems, including steroidal alkaloids, phenolic acids, steroidal glycosides, amino glycosides, polyamine alkaloids and amino sugars (Friedman, 2006; Chaparro et al., 2018, Joshi et al. 2020). These molecules may toxify or inhibit pathogen signaling pathways required for blackleg virulence (Joshi et al. 2015; Joshi et al. 2016; Li et al. 2014; Li et al. 2009; Naybi Muñoz-Cazares et al., 2017). However, very little is known about the genetics of blackleg resistant mechanism for any plant species (Chung et al. 2017; Joshi et al. 2020). As a result, breeding for resistance to blackleg in potato is difficult because the major genes involved have not been identified and resistance is most likely multigenic and quantitative (Zimnoch-Guzowska et al. 2000; Yogendra et al. 2014). Recently, a set of simple biochemical assays was shown to be associated with resistance to blackleg in potato, and metabolomic analysis suggests the identities of molecules involved in this resistance (Joshi et al. 2020). Identifying and validating the role of these molecules in resistance would facilitate rapid screening of potato varieties and provide a foundation for future breeding efforts.

Plants do not need to be resistant to pathogens, meaning that they limit pathogen growth, to be useful in agriculture. They may also be tolerant, meaning that pathogens grow and cause symptoms, but that sufficient yield is still obtained (Clarke 1986; Strauss and Agrawal 1999) (Little et al. 2010; Råberg 2014). The distinction between tolerant and resistant plants is clear for

many biotrophic and hemibiotrophic pathogens, but it is blurred for the soft rot pathogens, which can decay most plants under extreme anaerobiosis. For the purpose of this work, we use the term resistance for plants that remain asymptomatic, or nearly so, after inoculation with *Dickeya* or *Pectobacterium* in typical ambient temperature, humidity, and oxygen-level conditions that promote health potato growth.

Interestingly, accessions of some wild diploid potato species, such as *Solanum chacoense* are resistant to blackleg disease. *S. chacoense* has many advantages for adaption into potato breeding. For example, lines from these species tend to be vigorous, resistant to many common potato diseases, and they can be crossed with cultivated *S. tuberosum* (Jansky et al. 2014). Multiple draft or complete genomes are available for *S. chacoense* lines, including the complete genome of the self-compatible M6, which is a useful model *S. chacoense* and which is resistant to blackleg and soft rot compared to cultivated potato (Leisner et al. 2018; Felcher et al. 2012; Xu et al. 2011).

In this study, we compared metabolic profile of resistant and susceptible lines of *S. chacoens*e before and after inoculation with *D. dianthicola*. The objectives were (i) to provide a comprehensive overview of the stem metabolome of resistant and susceptible lines and (ii) to characterize biochemical differences in response to plant extracts to develop tools to screen for resistance, and (iii) to determine if a video-based assay could be used to identify resistant accessions. We used a video assay to screen 154 accessions of *S. chacoense* and identified two highly resistant and two highly susceptible lines. We found that plant extracts from the resistant lines inhibited virulence-related phenotypes, including quorum sensing (QS) and plant cell wall degarding enzymes (PCWDE). We also used metabolic profile to identify pre-formed and induced metabolites associated with resistance. These lines and the associated biochemical assays will

provide a foundation for future breeding efforts aimed at development of resistant potato cultivars and at understanding how quorum sensing inhibition affects the plant microbiome.

2. Materials and Methods

2.1. Plant propagation and maintenance

A total of 154 accessions of *S. chacoense* was obtained from (https://www.ars-grin.gov/Pages/Collections) and were screened against blackleg disease caused by D. dianthicola (ME23). Seeds of each accession were soaked in gibberellic acid (50 µg) overnight in 1 mL of sterile distilled water in centrifuge tubes. The seeds were then planted 0.5 cm below the surface of the potting mix (ProMix Bx General Purpose; Premier Tech Horticulture; Pennsylvania) in a 6.35 cm square form 103 pot (6.35 cm 6.35 cm 8.89 cm; L W H) and supplemented with the small amount of slow-release fertilizer "Osmocote Plus 15-9-12" (Scotts-MiracleGro; Ohio). The pots were placed into trays and covered with a closed container for one week. The trays were placed on a bench in a greenhouse room in Plant Growth Facilities (PGF) at Colorado State University. The pots were irrigated by hand once in three days for 1 month. The temperature in the greenhouse was set to 18-24 °C with a 16-hour daylight setting. A mixture of pesticides, including Botaniguard ES, Entrust SC, Molt-x, Distance, Judo, Avid, Compass, and 109 Azatin, were sprayed regularly for aphid control. Cuttings were made from one-month old plants to multiply the population.

2.2. Time-lapse video assay

To visualize disease development on wild potato lines, all 154 lines of *S. chacoense* were assessed using a time-lapse video assay. For each line, three plants were selected (three weeks old) grown in greenhouse as previously described. Prior to stem inoculation, *D. dianthicola* (ME23) cells were grown from a freshly frozen 20% glycerol stock on Nutrient Agar (NA) and incubated for 24 hours (hrs). A sterile toothpick was used to make a vertical slit about 2 cm above the soil

line. One loop of bacterial cells from NA plate was placed into the slit of the stem. Each wound was injected with 50 µL of 10^8 CFU/ml of *D. dianthicola* or 50 µL of sterile water (s H2O) as a negative control. The wounds were wrapped with Parafilm®, and the treated plants were placed in front of two cameras in an imaging room. An image was taken every 10 minutes for two weeks. All captured images were combined into a video file. The time from inoculation to first leaf wilt, 50% leaves wilting, 100% of leaves wilting were recorded and converted into hrs taken for each event to happen (Tylor et al. 2020). Two highly resistant and two highly susceptible accessions were selected for further analysis.

2.3. Stem inoculation for lesion length measurements

Overnight liquid cultures were started from a single colony of *D. dianthicola* and were grown in NB at 30°C for 12 hrs on an orbital shaker at 220 rpm. Bacteria were centrifuged and pellets were washed three times with sterile distilled water and adjusted to a final concentration of 1×108/mL in sterile water for stem inoculations. One-month old plants previously selected (two highly resistant and two highly susceptible) lines that were grown in the greenhouse were used for stem inoculations. A sterile toothpick was used to make a vertical slit on the lower stem of a plant about 2 cm from the soil. 50 µL of the bacterial suspension was injected into the stems using sterile pipette. Sterile water was injected in negative control samples. The wound was then wrapped with Parafilm ® and all treated plants were grown for three weeks in the greenhouse. Inoculated plants were screened based on the lesion length.

2.4. Sample preparation for metabolic profiling

One-month old plants of previously selected plants were used for stem inoculation. Samples were collected at two time points (0hrs and 24hrs) with three biological replications and three plants were pooled as one replication per treatment. 50 uL of the bacterial solution was

injected as previously described and the same amount of sterile water was injected in control samples. Subsequently, after each time point (0 hrs and 24 hrs), stems sections 1 cm above and 1 cm below the inoculated region were collected and flash-frozen in liquid nitrogen to quench the metabolites. The samples were stored at -80°C until they were lyophilized with a freeze drier for 48 hrs (HarvestRight, UT, USA). The freeze-dried stems were then used for metabolite extraction. For metabolite extraction, 1 mL of 6:3:1 methyl-tert-butyl ether/methanol/water (vol/vol/vol) was added to ~30 mg of tissue, agitated for 2 hrs at 4°C using a vortex machine, then sonicated for 30 min at cold water followed by vertexing on a shaker at 4°C for 1 hr. The mixture was the centrifuge for at 3,500 rpm for 15 mins at 4°C and the supernatant was transferred to a new vial and dry under a stream of nitrogen gas (Organomation Assocaition Inc., U.S.A.).

2.5. Liquid chromatography-mass spectrometry (LC-MS)

The LC-MS data was acquired in the phenylhexyl positive mode. A volume of 1.4 mL of organic extract from each stem sample was collected, dried down, and resuspended in 600 uL of 1:1 toluene/methanol. One microliter of the 10x diluted organic extract was injected onto a Waters Acquity UPLC system in randomized order with a pooled quality control (QC) injection after every six samples. Separation was achieved using a Waters Acquity UPLC CSH Phenyl Hexyl column (1.7 µM, 1.0 x 100 mm), using a gradient from solvent A (Water, 2mM ammonium formate) to solvent B (Acetonitrile, 0.1% formic acid). Injections were made in 99% A, held at 99% A for 1 min, ramped to 98% B over 12 minutes, held at 98% B for 3 minutes, and then returned to starting conditions over 0.05 minutes and allowed to re-equilibrate for 3.95 minutes, with a 200 µL/min constant flow rate. The column and samples were held at 65°C and 6°C, respectively. The column eluent was infused into a Waters Xevo G2-XS Q-TOF-MS with an electrospray source in positive mode, scanning 50-1200 m/z at 0.1 seconds per scan, alternating between MS (6 V collision

energy) and MSE mode (15-30 V ramp). Calibration was performed using sodium formate with 1 ppm mass accuracy. The capillary voltage was held at 700 V, source temperature at 140°C, and nitrogen desolvation temperature at 600°C with a desolvation gas flow rate of 1000 L/hr.

2.6. Data processing and metabolite annotation

For data processing, .RAW files were converted to .cdf format and processed according to Yao et al. 2019 in R statistical software (RCoreTeam, 2015). Peak detection, grouping, alignment, and filling was performed using XCMS in R (Smith et al. 2006), deconvoluted and normalized to total ion current using the RAMClust package in R (Broeckling et al. 2014). Interpretation of spectra occurred using the R package Interpret MS spectrum and MS-Finder v3.0 (Tsugawa et al. 2016; Lai et al. 2018), which determined molecular weights of fragments, chemical formulas and structures that were eventually matched to the external MS databases like, METLIN, MetFrag and NIST library search.

2.7. Biochemical assays

One-month old plants were inoculated with 1×10^8 cells of bacteria as described above. After 24 hrs, the stems were collected for all four accessions and ground with a sterile mortar and pestle. The stem extract was then centrifuged at 16,000 g for 1 min followed by filter sterilization. The filtered sterilized stem sap was centrifuged at 16,000 g for 5 min. The supernatant was used to test for inhibition of exoenzymes caused by *D. dianthicola*, including inhibition of pectate lyase (Pel), cellulase (Cel) and protease (Prt) activity. The exoenzyme activity was performed in the petri dish assays as described previously by Chatterjee et al., 1995.

To examine AHL activity, the reporter bacteria, CV026 (mini-Tn5 mutant in *luxI* homolog) was used to detect the presence of AHL molecules with the protocol described by Vijayaraghavan and Vincent, 2013. The reporter strain CV026 was grown in fresh NB medium supplemented with

kanamycin (50 μg/mL). Plant extracts were prepared as described previously. Bacteria were grown in the stem extract and the supernatants were filter sterilized and poured into central hole of LB plates (5 mm diameter). The CV026 strain was spread on the plate using microbial loop on four sides of the hole on LB plates. The plates were then incubated overnight at 30°C and the intensity of the purple pigment produced by the reporter strain was then assessed in cm^2.

2.8. Microscopy assays

Stem extracts of one month old resistant and susceptible lines were collected as mentioned above. Approximately 1×10^8 bacterial cells were added to the extract and grown at 30°C for 18 hrs on an orbital shaker at 220 rpm. The bacterial cultures were centrifuged for 5 mins at 16,000 rcf and pellets were washed three times with sterile distilled water. Bacterial cells were observed under compound microscope Nikon eclipse E400 using 1000X magnification.

2.9. Statistical analysis

The data for exoenzymes and time-lapse assays were analyzed using GraphPad software, version 8.0 (SAS Institute Inc., NC, USA) using Student's t tests to compare between treatments, with a p threshold of 0.05. Univariate and multivariate analyses were used to analyze the data. Spearman's correlations and hierarchical clustering was conducted to understand correlation patterns in treatments (resistant, susceptible and time course). Metabolite abundance and differences were evaluated using analysis of variance (ANOVA). Benjamini-Hochberg correction was used to adjust p-values for false discovery rate (FDR). Principle component analysis (PCA) and optical principle discriminate analysis (OPL-DA) of metabolites were performed on mean-centered and unit-variance scaled data using SIMCA v 14.1 (Umetrics, Umea, Sweden). Z- scores for metabolites were calculated using the relative abundance of metabolites compared to the mean and standard deviation of metabolites. Z scores were then used to generate a heatmap using

GraphPad Prism 8. Fold change (FC) for metabolites were calculated using the mean relative abundance values of resistant accessions compared to the mean susceptible abundance for the same metabolite.

3. Results

3.1. Virulence assay to rate Dickeya infection to S. chacoense lines

Among 154 lines of *S. chacoense* that were screened, 29 were scored as resistant, 105 intermediate and 20 were susceptible based on the development of disease symptoms including dark brown to black lesion, leaf wilting and plant death. Among the selected resistant and susceptible lines, disease symptoms were closely monitored, number of days taken to develop symptoms were recorded, and two highly resistant (R-1, accession no. PI 472819 and R-2, PI 230580) and two highly susceptible (S-1, PI 458319 and S-2, PI 320285) lines were selected for further analysis.

3.2. Lesion length and time-lapse video revealed that wilting symptoms develop faster in susceptible lines than in resistant lines

Dark brown or black lesions formed on plants from each accession when inoculated with *D. dianthicola* (ME23) after 48 hrs (Figure 4.1A). Lesions did not occur in controls inoculated with sterile distilled water. Among the four diploid potato lines screened, S-1 and S-2 had the longest lesions (MeanS-1 5.994 cm, MeanS-2 6.269 cm) and the lesions were significantly larger than those that formed on stems of R-1 and R-2 ($P < 0.05$). Lesion length results were correlated with the time-lapse video data. The two resistant lines took longer to develop wilting symptoms (MeanR-1, first leaf wilt = 98 hrs; MeanR-1, 50% leaf wilt = 300 hrs; MeanR-2, first leaf wilt = 80 hrs; MeanR-2, 50% leaf wilt = 320 hrs) (Figure 4.1B). R-1 and R-2 were significantly different from S-1 and S-2 in the video assay ($p < 0.05$). There were no significant differences observed

between R-1 and R-2, however R-1 developed smaller lesion compared to R-2. Infected plants of R-1 and R-2 survived up to 53 and 45 days respectively.

3.3. Metabolite extracts from resistant wild diploid potato lines reduced D. dianthicola exoenzyme activity and AHL synthesis

Stem extracts from the resistant and susceptible wild diploid potato lines were tested to measure enzyme activity. R1 and R2 stem extracts completely inhibited bacterial protease (Prt) and pectate lyase (Pel), and reduced cellulase (Cel), shown in Figure 4.2. We tested the effect of stem extracts on *D. dianthicola* production of AHL with the reporter strain *Chromobacterium violaceum* CV026. The R-1 stem extract completely inhibited *D. dianthicola* AHL production. Stem extract from potato line R-2 reduced AHL production compared to extracts from lines S-1 and S-2 (Figure 4.3). S-1 and S2 reduced AHL production compared to the negative control, demonstrating that they had some ability to reduce AHL production (not shown).

Stem extracts from resistant lines of *S. chacoense* altered *D. dianthicola* cell morphology and motility. *D. dianthicola* cells grown in stem extract from *S. chacoense* lines were heterogenous, with numerous elongated and filamentous cells compared to cells grown in NB. Some cells were rod-shaped, with length from 1 to 10 µm, and some cells were filamentous, with a length greater than 10 µm (Figure 4.4). The size of cells was significantly increased when grown in stem extracts of resistant and susceptible accessions compared to cells grown in NB. There was no significant difference observed in swimming and swarming motility of bacteria grown in resistant and susceptible stem extracts.

3.4. Metabolomic analysis of S. chacoense stem extracts using liquid chromatography mass spectrometry

A comparative metabolomics experiment was performed to identify metabolic profile associated with antibacterial activity in resistant lines (R-1 and R-2) compared to susceptible lines (S-1 and S-2). The metabolomic analysis detected a total 1,917 metabolites among four lines of *S. chacoense* (Figure 4.5A). Principle coordinate analysis (PCoA) was performed to distinguish differences between and among resistant and susceptible lines (Fig. 5A). We observed a clear separation between R-1 compared to S-1, S-2 and R-2 ($p < 0.0005$). Interestingly, R-1 and R-2 clustered separately suggesting that they might be resistant for different reasons, i.e. different metabolites are responsible for triggering resistance in both lines. The metabolomic profile were further evaluated using the regression model, orthogonal partial least square discriminate analysis (OPLS-DA). The OPLS-DA scores and loading plots demonstrated metabolic variation between the resistant and susceptible lines over the period of time. The R-1 model resulted in two OPLS-DA components that classified level of resistance, and component 2 separated the R-1 from two susceptible accessions (Fig 4.5B; R2Y component 22%). The R-2 model was also separated along components 2 (Figure 4.5C; R2Y component 2 23%). A total of 63 metabolites found were highly associated with resistance using these models, 32 were found in R1 model and 31 in R2 model.

Of 63 metabolites, we were able to annotate 17 metabolites with level 4 confidence and 21 with level 2 confidence. Among 17 metabolites, 15 were reported previously to exhibit antibacterial activity against pathogens in plants. Interestingly, nine annotated metabolites were previously tested to show strong antibacterial activities by interfering with QS related genes, seven were from R-1, including capsaicin, salicylic acid, salicylamide, tomatidine, indole 3-carbinol, menthol, naphthoquinone shikonin, 2-heptyl-3-hydroxy-4-quinolon and two from R-2,

10′(Z),13′(E)-heptadecadienylhydroquinone and maleic acid. Detailed information of metabolites that were highly associated with resistance is provided in Table 4.1.

Two metabolites were associated with resistance in both R-1 and R-2 OPLS-DA models, belonging to benzenoids and alkaloids families, and their metabolic abundance was decreased in susceptible accessions (Figure 4.5D). In many cases, the mechanisms these families deploy to inhibit pathogen are well explored. For instance, they inhibit QS related genes such as *rhII* and p*qsA*, *expI* and *expR* (Borges et al. 2014; O'May and Tufenkji 2011, Joshi et al. 2020). These findings suggested that resistant lines inhibit QS dependent mechanisms in blackleg pathogens

3.5. Induction of S. chacoense metabolites after inoculation with D. dianthicola.

To increase our understanding of innate or pathogen triggered immunity (PTI) in resistant wild diploid potatoes lines, we compared metabolic profile of resistant (R1 and R2) and susceptible (S1 and S2) lines at 0 and 24 hrs post inoculation. Surprisingly, we observed a very little shift in metabolic abundance between the treatments (Figure 4.6A, Table 4.2). To further evaluate the difference, fold change and pairwise comparisons were conducted to quantify the magnitude of differences between resistant and susceptible lines (Figure 46B, 4.6C, Table 4.2). We found 43 metabolites that were constant in inoculated resistant lines compared to the susceptible lines (Figure 4.6C, Table 4.2).

Of 43 metabolites, the majority are associated with nitrophenols, tannins, flavonoids and sesquiterpenes in resistant lines before infection, however phenylpropanoids, diterpenoids and furanoid lignan were found in infected resistant lines. These metabolites are reported to interfere with bacterial virulence systems and to attenuate disease (Kowalczyk et al. 2015; Redondo et al. 2014; Tomiyama et al. 2016; Górniak et al. 2019; Rukayadi and Hwang 2006; Konno et al. 1990).

4. Discussion

We screened 154 accessions of the diploid potato *S. chacoense* to identify lines that were either highly resistant, moderately resistant or highly susceptible to blackleg caused by *D. dianthicola* (Supplementary Table 4.3). We chose two resistant and two susceptible lines for further bio-chemical assays and found that the resistant lines inhibited quorum sensing and production of enzymes regulated by quorum sensing, with R-1 inhibiting activity to a greater extent than R-2. We also found that the metabolic profiles of the resistant lines differed from the susceptible lines and from each other. We identified metabolites in resistant lines that were previously reported to inhibit quorum sensing activity of plant pathogens.

Of the four lines examined in the present study, we found significant differences in disease development and lesion length in resistant lines compared to susceptible lines. Interestingly, in both cases, R-1 was more resistant than R-2. The lesion length produced by *D. dianthicola* in R-1 was smaller and infected plants survived and flowered up to 52 days after infection compared to R-2 average to 45 days. R-1 appeared to suppress quorum sensing and associated phenotypes to a greater extent than R-2, but the differences were not significantly different. As with Joshi et al. (2020), our data supports the hypothesis that these *S. chacoense* plants resist blackleg through suppression of quorum-sensing through plant metabolites that either interfere with AHL production or sensing. For example, the plant defense hormone salicylic acid and its derivative, salicylamide both were found at higher levels in R-1 and these molecules are known to interfere with production of AHL through inhibition of the acyl homoserine lactone synthase ExpI (Joshi et al. 2020).

These results are similar to those reported by Joshi et al. (2020) for *S. chacoense* M6, demonstrating that multiple blackleg-resistant *S. chacoense* lines have similar effects on inhibition

of quorum sensing and the virulence enzymes regulated by quorum sensing. Our study differs in that we used *D. dianthicola* instead of *Pectobacterium brasiliense*, and because we identified susceptible *S. chacoense* lines. The susceptible lines described here and in Joshi et al. (2020) demonstrate that susceptibility is correlated with lack of QS inhibition in both *S. chacoense* and *S. tuberosum*. These susceptible *S. chacoense* lines will be useful for mapping of genes responsible for metabolites that inhibit quorum sensing. In addition, we also used a different metabolite extraction method and found unique compounds in resistant lines known to interfere with quorum sensing including benzenoids (salicylic acid, salicylamide, capsaicin) and alkaloids (Indole 3 carbinol, tomatidine) not reported by Joshi et al. (2020). Both extraction methods result in unique identification of compounds that are reported to exhibit anti-bacterial properties.

Plant defense responses against pathogen could be either pre-formed (phytoanticipins) or induced (phytoalexins) (VenEtten et al. 1994; Müller & Börger 1940; Pedras and Yaya 2015). In both cases, these are plant-derived small molecules with diverse functions in plant defense responses (Davey and O'toole 2000; Hall-Stoodley et al. 2004). We found very little to no shift in metabolic abundance of specific metabolites when we compared infected with non-infected at time point 0 versus 24 hrs post infection (Figure 4.6A), suggesting that if metabolites contribute to resistance to blackleg, that it occurs through pre-formed metabolites. These finding support previous work where researchers found that potato has constitutive defense against pathogens (Ali et al. 2012).

SRP use several virulence factors to cause disease such as quorum sensing, bacterial biofilm formation, motility, toxins, pigments, enzymes, and surfactants. Conversely, plants produce thousands of metabolites to overcome diseases caused by pathogens (Wang et al. 2019). Our metabolic analysis revealed that resistance in wild diploid potatoes is tightly linked with QS

inhibiting molecules. Consistent with Joshi et al. 2020, we found molecules that were previously reported to exhibit stronger antibacterial activity by inhibiting quorum sensing related genes. For instance, capsaicin, salicylic acid, tomatidine, indole 3-carbinol, menthol, naphthoquinone shikonin, 2-heptyl-3-hydroxy-4-quinolon, 10′(Z),13′(E)-heptadecadienylhydroquinone have been previously listed to inhibit QS activity in Gram-negative bacteria (Qiu et al. 2012; Ding et al. 2011; Monte et al. 2014; Liu et al. 2012; Qiu et al. 2011; Qiu et al. 2010; Husain et al. 2015; Gutiérrez-Barranquero et al. 2015; McKnight et al. 2000; Diggle et al. 2007). These metabolites with antimicrobial or anti-virulence properties may contribute to blackleg resistance by inhibiting QS and hence PCWDEs productions or activity. It is possible that the disruption of bacterial QS led to attenuations of resistance against blackleg disease in resistant lines (Kaufmann et al. 2008; Smith and Iglewski 2003; Jhoshi et al 2016). We also found plant defense hormone salicylic acid and its derivative salicylamide in R-1. Hight levels of constitutive salicylic acid in potato plants have been previous reported, it directly binds to *Pectobacterium*- acyl-homoserine lactone synthesis and reduce QS (Joshi et al. 2020)

Cultivated potato, *S. tuberosum*, is an outcrossing tetraploid with limited information available for genetic or genomic studies compared to other major crops. A major limitation in understanding and using genetic resistance to blackleg is that it is multigenetic, quantitative, and difficult to screen for if only virulence assays are used (Charkowski et al. 2018; Corwin et al. 2017). The observation that QS inhibition is correlated with resistance will increase efficiency in screening for blackleg resistance in potato breeding programs. The QS inhibition screen combined with diploid mapping populations may allow plant scientists to identify the specific genes required for QS inhibiting metabolites.

Although the blackleg pathogens *Dickeya* and *Pectobacterium* produce numerous plant cell wall degrading enzymes, they do not appear to cause epidemics outside of agricultural ecosystems, suggesting that most plants resist these pathogens. Our finding aids in exploring genotypes with constitutive active defense that can be tracked with simple AHL detection assays. This germplasm and the AHL assays may be useful tool for breeders and farmers to screen for resistant against blackleg disease. Further investigation of monitoring the segregation of these resistant genetic attributes in progeny will provide insights to genes important for production of AHL inhibitors and may guide and promote development of elite cultivars with durable resistance against plant pathogens.

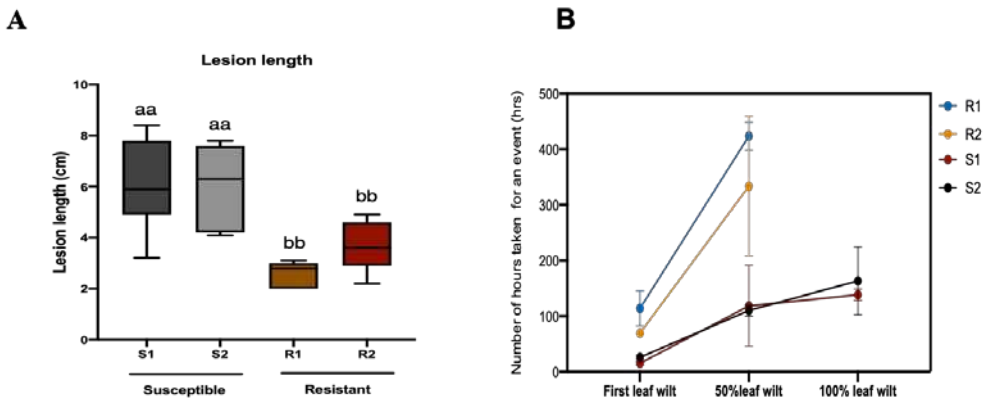


Figure 4.1. Virulence assay to rate *D. dianthicola* infection to *S. chacoense* lines. A, Box plot of lesion length (cm) measured on four lines of *S. chacoense* after infection with *D. dianthicola* ME23. Lesions were not observed from wounded plant control. Different letters represent statistically significant. B, the average time taken ± standard deviations (hrs) for developing blackleg symptoms after resistant and susceptible lines were injected 10^8 cells of *D. dianthicola*. (* P < 0.05).

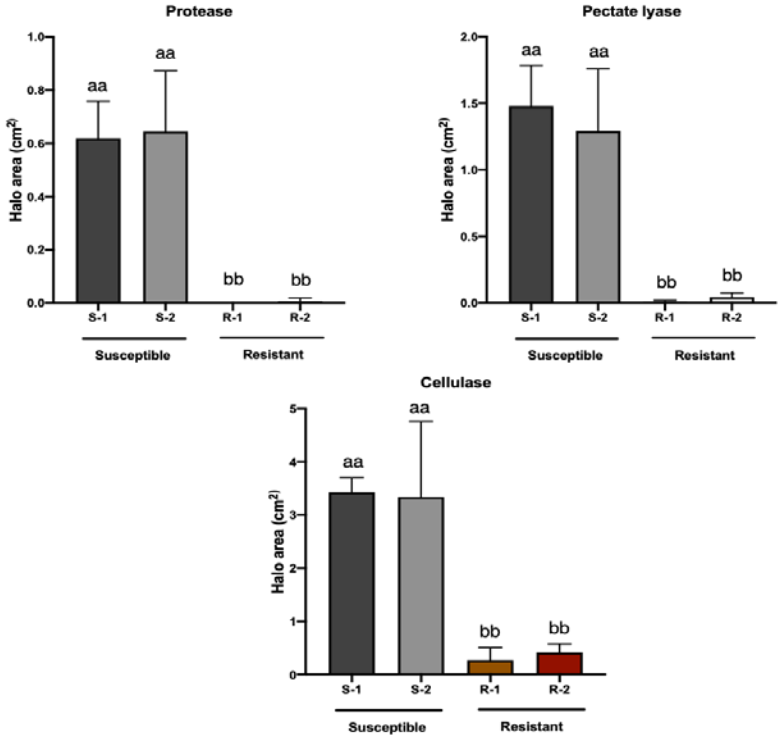


Figure 4.2. Effects of resistant and susceptible lines stem extract on exoenzyme activity of *D. dianthicola* ME23. Enzyme activity assays after growing bacterial cells approximately 10^8 cells in stem extract for 12 hrs at 28°C under continuous shaking. Different letters indicate difference between resistant and susceptible lines (ANOVA, $P<0.05$).

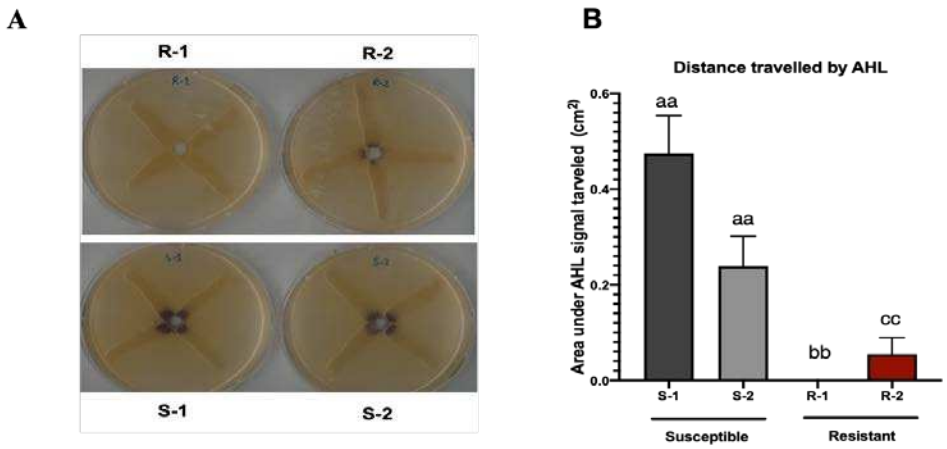


Figure 4.3. Effects of resistant and susceptible lines extract on N-acyl homoserine lactone (AHL) of *D. dianthicola* ME23. A, Purple pigment exhibited by CV026 as a response of AHL present in *D. dianthicola* grown supernatant. Pictures were taken after incubating the plates at 30°C for 24 hrs. B, bar plot showing the distance travelled by AHL ($cm^{2)}$. Different letters indicate the differences between resistant and susceptible stem extract (ANOVA, $P<0.05$). The data shown are from one of three repetitions and are representative of all repetitions of this experiment.

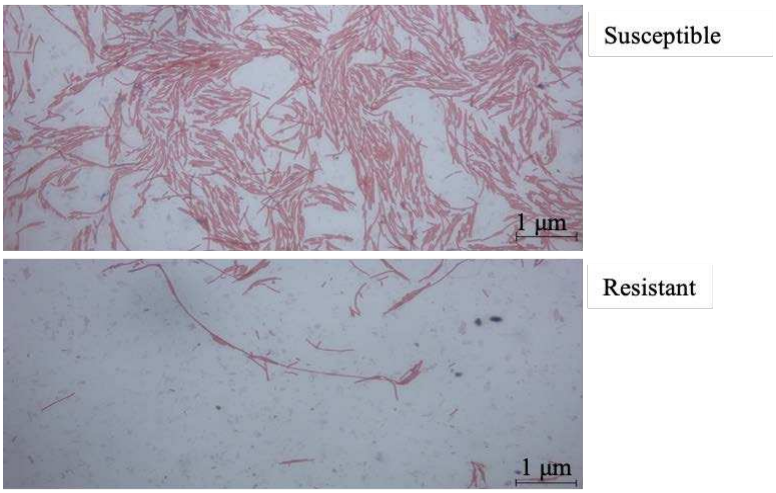


Figure 4.4. Effect of resistant and susceptible stem extracts on morphology of *D. dianthicola* ME23.

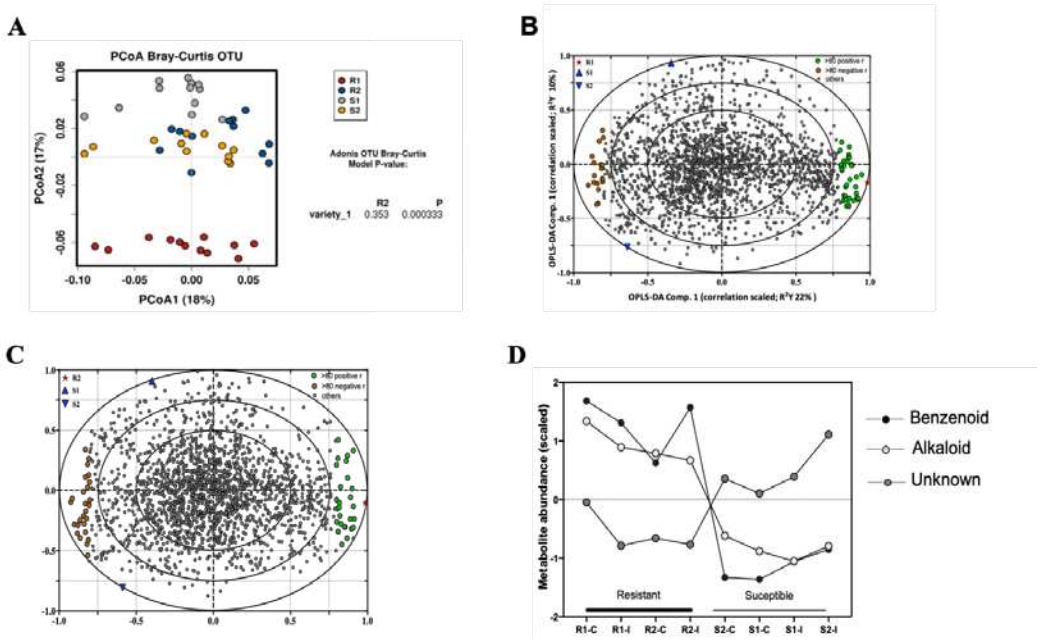


Figure 4.5. Metabolic response over time to *D. dianthicola* ME23 infection in resistant and susceptible potato lines. A, Principal component analysis of based on LC-MS molecular features for a total n = 1,917 metabolites-based data points. Orthogonal projection to latent structures discriminant analysis was performed within the B, R-1 compared to S-1 and S-2 and C, R-2 compared to S-1 and S-2 based on resistance and susceptibility. Symbols (star, triangle) represents represent correlation-scaled mean scores. D, Stem metabolites associated resistance and susceptibility were z transformed (scaled).

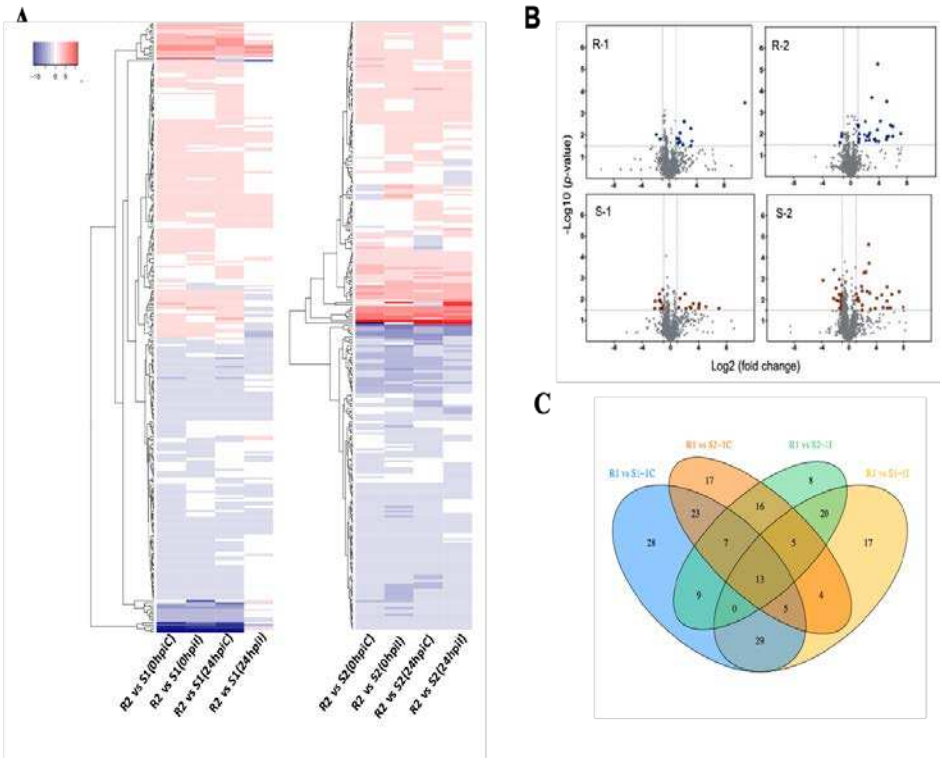


Figure 4.6. Metabolic response over time to *D. dianthicola* infection and control in stems of potato lines. A, Heat map of 661 metabolites. Bars represent $\log_2$ FC with P<0.05 was used for each comparison at 0 and 24hrs post inoculation and infected vs control. B, Volcano plot for differential abundance ($\log_2$, x-axis) and significance ($-\log_{10}$ *P* value, y-axis of 1,917 metabolites (gray dots). C, Venn diagram representing metabolites which are consistent in each comparison base on heatmap.

Table 4.1. Potato stem metabolites associated with inhibiting virulence of *D. dianthicola* ME23

	Super-class[aa]	Sub-class[ab]	Metabolites[ac]	Identifier[b]	R1 vs S1[ca]	R1 vs S2[cb]	R2 vs S1[cc]	R2 vs S2[cd]	Reported antimicrobial activity
1	Benzenoids	Benzenesulfonamides	Phenol, 4-phenylamino	C0015	0.71	0.61	0.28	0.18	
2		Methoxyphenols	Capsicin	C0020	0.66	0.63	0.59	0.56	Qiu et al. 2012*
3		Naphthalene sulfonic acids and derivatives		C0022	1.3	0.96	1.24	0.89	
4		hydroxy-4-unsubstituted benzenoids/ Phenylmethylamines	Salicylamide	C0465	2.77	2.18	2.9	2.31	Lin et al. 2012 *
5		Benzoic acids and derivatives	salicylic acid	C1242	1.37	1.29	-2.13	-2.21	Monte et al. 2014; Bandara et al. 2006; Chang et al. 2014; Joshi et al. 2020 *
6		Benzoic acids and derivatives	Methyl 3,5-bis(octadecyloxy)benzoate	C0290	-0.27	-0.17	0.92	1.02	
7		Anthraquinones	1,2-Benzenedicarboxylic acid	C0951	-0.94	-2.62	2.55	0.88	
8		Benzenediols	Isoquinoline, 10′(Z),13′(E)-Heptadecadienylhydroquinone	C0533	0.22	-1.12	1.51	0.17	Liu et al. 2012 *
9	Alkaloid	Alkaloids and derivatives	Rescinnamine	C0716	3.07	1.45	-1.25	-2.87	

10		Alkaloids and derivatives	Tomatidine	C0134	-0.23	-0.49	0.73	0.47	Rutherford and Bassler 2012; Mitchell et al. 2012; Ji et al. 1995 *
11		Alkaloids and derivatives	α-solamirine	C0140	0.12	0.15	0.6	0.62	et al. 2014 *
12		Alkaloids and derivatives	Indole 3 carbinol	C0360	-0.33	-0.25	1.55	1.63	Monte et al. 2014; Lee et al. 2012*
13	Lipids and lipid like molecules	Glycerophosphates		C0012		0.62	0.72	1.01	0.7
14		Glycerophosphocholines		C0247	0.82	0.82	0.8	0.8	Hussein et al. 2019*
15		Glycerophosphocholines		C0608	0.85	0.78	0.37	0.3	
16		Glycerophosphocholines		C0733	2.49	2.21	0	-0.28	
17		Glycerophosphocholines		C1655	6.28	6.4	0.55	0.67	
18		Glycerophosphocholines		C0566	-0.11	-0.21	1.05	0.95	
19		Polyprenols		C0282	1.23	1.79	1.43	1.99	Tao et al. 2016**
20		Polyprenols		C0806	2.01	1.87	0.18	0.04	Tao et al. 2016**
21		Glycerophosphoserines		C0469	1.23	1.87	-0.17	0.48	

22	Glycerophosphoserines		C0580	1.23	1.86	-0.49	0.15	
23	Glycerophosphoserines		C1281	1.4	2.03	-0.01	0.62	
24	Glycerophosphoethanolamines		C0775	1.44	1.34	1.64	1.55	
25	Glycerophosphoethanolamines		C0697	-1.99	-1.2	-0.02	0.77	
26	Triterpenoids/Terpene glycosides		C1214	2.86	-0.24	4.37	1.27	Mahizan et al. 2019*
27	Triterpenoids		C0839	0.12	-1.42	1.69	0.16	Cunha et al. 2010 Barbieri et al. 2017**
28	Triterpenoids		C1222	-0.51	-2.47	3.75	1.79	
29	Triterpenoids		C1223	1.94	0.81	3.84	2.7	
30	Terpene glycosides		C0297	1.49	-1.17	3.22	0.56	Mahizan et al. 2019**
31	Glycosphingolipids		C1746	1.03	0.97	0.79	0.73	Aerts et al. 2019**
32	Glycosphingolipids/Hopanoids		C0094	-0.41	-0.51	1.26	1.16	
33	Steroidal glycosides	cholesteryl 6-O-oleoyl-beta-D-galactoside	C0512	0.09	-0.92	1.45	0.44	

34		Glycerophosphoethanolamines		C0679	-0.31	-0.67	1.14	0.78	
35	Phenylpropanoids and polyketides	Coumarins and derivatives	Coumarin, 2-heptyl-3-hydroxy-4-quinolone	C1211	5.82	1.82	4.37	0.37	Gutiérrez-Barranquero et al. 2015; McKnight et al. 2000; Diggle et al. 2007*
36		Zearalenones	(S,E)-Zearalenone	C0961	0.55	0.04	4.36	3.85	Truchado et al. 2012**
37		Macrolides and analogues	21-Hydroxyoligomycin A_1	C045	0.02	0.28	-0.13	0.14	Shryock et al. 1998**
38	Organoheterocyclic compounds	aloquinolines		C0052	0.78	0.1	1.05	0.37	
39		Benzothiadiazines/ Hydropyridines		C0952	-2.08	-2.92	1.01	0.17	
40	Organic acids and derivatives	Dicarboxylic acids and derivatives	Maleic acid	C0111	-0.13	-0.73	0.72	0.13	
41	Organic oxygen compounds	Carbonyl compounds	Salicylamide	C1638	1.98	1.81	-0.21	-0.38	
42	Hydrocarbons	Alkanes	Octacosane	C0498	-0.16	-0.32	0.85	0.7	
43	Sphingolipids			C1350	2.45	0.64	1.77	-0.05	
44	Unknowns	Unknown	[M +H]						

45		C0555	1.59	0.9	0.47	-0.22
46		C0872	3.81	3.83	-0.61	-0.59
47		C1641	2.24	1.37	1.6	0.72
48	822.642	C0181	-0.28	-0.3	0.83	0.8
49	855.58	C1112	2.2	1.17	1.35	0.32
50	764.52	C1138	0.64	0.84	0.91	1.11
52	766.536	C0446	1.51	0.61	1.49	0.58
53		C0590	1.67	2.62	3.44	4.39
54	825.533	C1138	0.64	0.84	0.91	1.11
55	825.533	C1260	2.51	1.81	1.75	1.06
57	745.557	C0905	0.38	0.3	0.58	0.5
58	575.21	C0946	3.41	1.76	1.67	0.02

59	732.445	C0981	3.3	3.28	0.76	0.73
60	411.362	C1831	0.59	-0.58	1.74	0.57
61	640.587	C1846	0.37	0.25	1.81	1.69
62	607.215	C0425	1.29	1.17	1.07	0.95
63	411.764	C1789	0.8	0.88	0.39	0.48

[aa, ab, ac] Metabolite annotation based on interpretation of mass spectrometry data. Level-3 annotation (denotated by super/sub-class) when spectrum indicate a chemical class but could not be confidently assigned a metabolite. M =natural mass and nd = annotation could not be determined.
[b] Identification number of the metabolite
[ca, cb, cc,cd] Log_2 fold-change(FC) values of resistant and susceptible lines comparisons
[d] Previously reported antimicrobial activity
[*]Metabolite reported to exhibit antimicrobial activity
[**] Metabolic family reported to exhibit antimicrobial activity

Table 4.2. List of metabolites consistent in resistant line. (A) Before infection, (B) after infection

(A)

	Super-class[aa]	Sub-class[ab]	Metabolites[ac]	Identifier[b]	R1 vs S1[ca]	R1 vs S2[cb]	R2 vs S1[cc]	R2 vs S2[cd]	Reported antimicrobial activity[d]
1	Benzenoids	Nitrophenols	Phenol, 2,5-dinitro-	C0042	1.87	1.39	1.21	0.73	Kowalczyk et al. 2015**
2		Diphenylmethanes	Hexachlorophene or	C0390					
3		Phenol esters	Phenol, 4-(phenylamino	C0472	1.98	1.9	0.61	0.52	Walsh et al. 2019**
4	Phenylpropanoids and polyketides	Tannins	Kadsuphilol L	C0942	4.91	3.17	2.78	1.03	Redondo et al. 2014; Tomiyama et al. 2016**
5		Flavonoids	Ikarisoside D_1	C0949	1.49	1.13	1.19	0.83	Górniak et al. 2019**
6		Flavonoids	Ikarisoside D_1	C0999	1.6	1.4	1.5	2.9	
7	Lipids and lipid-like molecules	Sesquiterpenoids	Nivalenol_2	C0096	-1.64	-0.96	-0.92	-0.24	Astani et al. 2010; Torres-Romero et al. 2011; Gomes et al. 2009; Rukayadi and Hwang 2006**

8		Fatty acids and conjugates	erythro-Canabisine H	C0192	3.94	2.73	0.61	-0.6	
9		Polyprenols	Solanesol	C0806	2.01	1.87	0.18	0.04	
10		Glycosphingolipids	Galabiosylceramide (d18:1/9Z-18:1)	C1774	0.98	2.18	-0.51	0.69	
11	Organic acids and derivatives	Amino acids, peptides, and analogues	Lissoclinamide 5_2	C1852	3.57	2.79	0.15	-0.63	Joshi et al. 2020**
12		Amino acids, peptides, and analogues	Lissoclinamide 5_3	C1860	3.7	1.63	1.17	-0.9	
13		Carbohydrates and carbohydrate conjugates	Linckoside C;(-)-Linckoside C_1	C0226	0.44	0.98	-0.38	0.16	
14		Amino acids, peptides, and analogues	Lissoclinamide 5_1	C1195	4.01	2.56	0.8	-0.64	
15	Alkaloids and derivatives		Syrosingopine 1	C0098	0.33	0.14	-0.63	-0.83	Parai et al. 2018*
16			Syrosingopine 2	C0540	2.16	2.22	0.09	0.15	
17			Brucine (A)	C1879	3.13	2.3	-0.76	-1.58	
18	Unknown			C0716	3.07	1.45	-1.25	-2.87	
19				C0373	0.55	1.27	-0.65	0.07	

20				C0753	1.53	1.5	0.21	0.19	
21				C0734	1.17	1.72	-0.35	0.19	
22				C0946	3.41	1.76	1.67	0.02	
23				C0981	3.3	3.28	0.76	0.73	

(B)

	Super-class	**Sub-class**	**Metabolites**	**Identifier**	**R1 vs S1**	**R1 vs S2**	**R2 vs S1**	**R2 vs S2**	**Reported antimicrobial activity**
1	Benzenoids	Methoxyphenols	[8]-Shogaol	C1305	1.39	1.16	-0.87	-1.1	Walsh et al. 2019; Yang et al. 2016 **
2		Phenylphosphines and derivatives	Diphenyl(4-tolyl)phosphine						Molodykh et al. 1983**
3	Organic acids and derivatives	Hybrid peptides	4-L-Serine-pepstatin A;Hydroxypepstatin A_1	C0580	1.23	1.86	-0.49	0.15	
4		Chlorins		C0870	3.09	2.53	-0.85	-1.4	
5		Alcohols and polyols		C0595	1.1	1.19	1.33	1.42	
6		Organoheterocyclic compounds		C1260	2.51	1.81	1.75	1.06	

7	Phenylpropanoids and polyketides	Macrolides and analogues	21-Hydroxyoligomycin A_1	C0425	1.29	1.17	1.07	0.95	Kanoh and Rubin 2010**
8		Hydroxycoumarins	Coumarin 1	C1211	5.82	1.82	4.37	0.37	Reen et al. 2018; Yang et al. 2016**
9		IsoflavHydroxycoumarinsonoids	Coumarin 2	C1212	-2.26	-0.14	-0.43	1.69	
10	Lipids and lipid-like molecules	Diterpenoids	Diacarnoxide C	C1617	1.37	0.08	-0.07	-1.36	Mahizan et al. 2019; Sadowska et al. 2016**
11		Stigmastanes and derivatives	28-Homocastasterone;24-S-24-Ethylbrassinone	C0543	0.44	0.16	-0.74	-1.02	
12		Glycerophosphoserines		C0938	1.11	1.99	0.46	1.34	
13		Glycosylglycerols	Oligomycin A	C1132	1.16	1.56	0.43	0.83	
14		Lineolic acids and derivatives	Cucurbic acid	C0572	-0.14	0.71	-1.71	-0.87	Jung et al. 2014; Magesh et al. 2013
15	Alkaloids and derivatives	Yohimbine alkaloids	Syrosingopine	C0540	2.16	2.22	0.09	0.15	
16		Yohimbine alkaloids	Methoserpidine	C0429	3.07	2.75	-0.28	-0.61	
17	Organic acids and derivatives	Pentacarboxylic acids and derivatives		C1220	2.14	1.94	-0.22	-0.42	Konno et al. 1990**

18		Carbohydrates and carbohydrate conjugates	C0716	3.07	1.45	-1.25	-2.87
19		Carbohydrates and carbohydrate conjugates	C0716	3.07	1.45	-1.25	-2.87
20	Unknown		C0582	0.55	1.41	-0.67	0.19

[aa, ab, ac] Metabolite annotation based on interpretation of mass spectrometry data. Level-3 annotation (denotated by super/sub-class) when spectrum indicate a chemical class but could not be confidently assigned a metabolite. M =natural mass and nd = annotation could not be determined.
[b] Identification number of the metabolite
[ca, cb, cc,cd] Log_2 fold-change(FC) values of resistant and susceptible lines comparisons
[d] Previously reported antimicrobial activity
[*]Metabolite reported to exhibit antimicrobial activity
[**] Metabolic family reported to exhibit antimicrobial activity

REFERENCES

Aerts, J. M. F. G., Artola, M., van Eijk, M., Ferraz, M. J., and Boot, R. G. 2019. Glycosphingolipids and infection. potential new therapeutic avenues. Front. Cell Dev. Biol. 7.

Agnew, P., C Koella, J., and Michalakis, Y. 2000. Host life history responses to parasitism. Microbes Infect. 2:891–896.

Ajani, O. O., Familoni, O. B., Echeme, J. O., Wu, F., and Sujiang, Z. 2013. Synthesis and antibacterial activity of N,N- diethylamide bearing benzenesulfonamide derivatives. J. Am. Chem. Soc. 3:34-39.

Ali, A., Moushib, L. I., Lenman, M., Levander, F., Olsson, K., Carlson-Nilson, U., et al. 2012. Paranoid potato. Plant Signal Behav. 7:400–408.

Allison, D., Delancey, E., Ramey, H., Williams, C., Alsharif, Z. A., Al-khattabi, H., et al. 2017. Synthesis and antimicrobial studies of novel derivatives of 4-(4-formyl-3-phenyl-1H-pyrazol-1-yl) benzoic acid as potent anti-Acinetobacter baumanni agents. Bioorganic Med. Chem. Lett. 27:387–392.

and biofilm cells of *Staphylococcus epidermidis*. Curr Microbiol. 59:118–122.

Arora, R. K. 2012. Eco-friendly management of soil and tuber borne diseases of potato. Indian Phytopathol. 65:116–21.

Astani, A., Reichling, J., and Schnitzler, P. 2010. Comparative study on the antiviral activity of selected monoterpenes derived from essential oils. Phytother Res. 24:673–679.

Bai, J., Wu, Y., Liu, X., Zhong, K., Huang, Y., and Gao, H. 2015. Antibacterial activity of shikimic acid from pine needles of Cedrus deodara against *Staphylococcus aureus* through damage to cell membrane. Int. J. Mol. Sci. 16:27145-27155.

Bandara, M. B. K., Zhu, H., Sankaridurg, P. R., and Willcox, M. D. P. 2006. Salicylic acid reduces the production of several potential virulence factors of *Pseudomonas aeruginosa* associated with microbial keratitis. Invest. Ophthalmol. Vis. Sci. 47:4453–4460.

Barbieri, R., Coppo, E., Marchese, A., Daglia, M., Sobarzo-Sánchez, E., Nabavi, S. F., et al. 2017. Phytochemicals for human disease: An update on plant-derived compounds antibacterial activity. Microbiol Res. 196:44–68.

Barnard, A. M. L., Bowden, S. D., Burr, T., Coulthurst, S. J., Monson, R. E., and Salmond, G. P. C. 2007. Quorum sensing, virulence and secondary metabolite production in plant soft-rotting bacteria. Phlios Trans R Soc B Biol Sci. 362:1165–1183.

Boer, S. H. D., Boucher, A., and Haan, T. L. D. 1996. Validation of thresholds for serological tests that detect *Clavibacter michiganensis* subsp. *sepedonicus* in potato tuber tissue. EPPO Bulletin. 26:391–398.

Bolton, M. D. 2009. Primary metabolism and plant defense-fuel for the fire. Mol. Plant. Microbe Interact. 22:487–497.

Borges, A., Ferreira, C., Saavedra, M. J., and Simo˜es, M. 2013. Antibacterial activity and mode of action of ferulic and gallic acids against pathogenic bacteria. Microb. Drug Resist. 19:256-265.

Broeckling, C. D., Afsar, F. A., Neumann, S., Ben-Hur, A., and Prenni, J. E. 2014. RAMClust: A novel feature clustering method enables spectralmatching-based annotation for metabolomics data. Anal. Chem. 86: 6812-6817.

Chang, C.-Y., Krishnan, T., Wang, H., Chen, Y., Yin, W.-F., Chong, Y.-M., et al. 2014. Non-antibiotic quorum sensing inhibitors acting against N -acyl homoserine lactone synthase as druggable target. Sci Rep. 4:7245.

Chang, C.-Y., Krishnan, T., Wang, H., Chen, Y., Yin, W.-F., Chong, Y.-M., et al. 2014. Non-antibiotic quorum sensing inhibitors acting against N -acyl homoserine lactone synthase as druggable target. Sci. Rep. 4:7245.

Chaparro, J. M., Holm, D. G., Broeckling, C. D., Prenni, J. E., and Heuberger, A. L. 2018. Metabolomics and ionomics of potato tuber reveal an influence of cultivar and market class on human nutrients and bioactive compounds. Front. Nutr. 5:36

Charkowski, A. O. 2007. The soft rot Erwinia. Springer. 423–505.

Charkowski, A. O. 2015. Biology and control of *Pectobacterium* in potato. Am. J. Potato Res. 92:223–229.

Charkowski, A. O. 2018. The changing face of bacterial soft-rot diseases. Annu. Rev. Phytopathol. 56:269–288.

Charkowski, A., Blanco, C., Condemine, G., Expert, D., Franza, T., Hayes, C., Hugouvieux-Cotte-Pattat, N., et al. 2012. The role of secretion systems and small molecules in soft-rot Enterobacteriaceae pathogenicity. Annu. Rev. of Phytopathol. 50:425–449.

Chatterjee, A., Cui, Y., Liu, Y., Dumenyo, C. K., and Chatterjee, A. K. 1995. Inactivation of rsmA leads to overproduction of extracellular pectinases, cellulases, and proteases in *Erwinia carotovora* subsp. *carotovora* in the absence of the starvation/cell density-sensing signal, N-(3-oxohexanoyl)-L-homoserine lactone. Appl. Environ. Microbiol. 61:1959-1967.

Chung, Y. S., Kim, C., and Jansky, S. 2017. New source of bacterial soft rot resistance in wild potato (*Solanum chacoense*) tubers. Genet. Resour. Crop Evol. 64:1963–1969.

Clarke, D. D. 1986. Tolerance of parasites and disease in plants and its significance in host-parasite interactions. Adv. Plant Pathol. 5:161-197.

Corwin, J. A., and Kliebenstein, D. J. 2017. Quantitative resistance: more than just perception of a pathogen. The Plant Cell. 29:655–665.

Cui, Y., Chatterjee, A., Yang, H., and Chatterjee, A. K. 2008. Regulatory network controlling extracellular proteins in Erwinia carotovora subsp. carotovora: FlhDC, the master regulator of flagellar genes, activates *rsmB* regulatory RNA production by affecting *gacA* and *hexA* (*lrhA*) expression. J. Bacteriol. 190:4610– 4623.

Cunha, W. R., de Matos, G. X., Souza, M. G. M., Tozatti, M. G., Andrade e Silva, M. L., Martins, C. H. G., et al. 2010. Evaluation of the antibacterial activity of the methylene chloride extract of *Miconia ligustroides*, isolated triterpene acids, and ursolic acid derivatives. Pharm. Biol. 48:166–169.

Cushnie, T. P. T., Cushnie, B., and Lamb, A. J. 2014. Alkaloids: An overview of their antibacterial, antibiotic-enhancing and antivirulence activities. Int. J. Antimicron. Agents. 44:377–386.

Cushnie, T. P. T., Cushnie, B., and Lamb, A. J. 2014. Alkaloids: an overview of their antibacterial, antibiotic-enhancing and antivirulence activities. Int. J. Antimicrob. Agents. 44:377-386.

Czajkowski, R., Perombelon, M. C., van Veen, J. A., and van der Wolf, J. M. 2011. Control of blackleg and tuber soft rot of potato caused by *Pectobacterium* and *Dickeya* species: a review. Plant pathol. 60:999–1013.

Diggle, S. P., Matthijs, S., Wright, V. J., Fletcher, M. P., Chhabra, S. R., Lamont, I. L., et al. 2007. The *Pseudomonas aeruginosa* 4-quinolone signal molecules HHQ and PQS play multifunctional roles in quorum sensing and iron entrapment. Chem. Biol. 14:87–96.

Ding, X., Yin, B., Qian, L., Zeng, Z., Yang, Z., Li, H., et al. 2011. Screening for novel quorum-sensing inhibitors to interfere with the formation of *Pseudomonas aeruginosa* biofilm. J. Med. Microbiol.60:1827–1834.

Douches, D.S., K. Jastrzebski, D. Maas and R.W. Chase. 1996. Assessment of potato breeding progress in the United States over the last century. Crop Sci. 36:1544-1552.

Essarts, Y. R. des, Cigna, J., Quêtu-Laurent, A., Caron, A., Munier, E., Beury-Cirou, A., et al. 2016. Biocontrol of the potato blackleg and soft rot diseases caused by *Dickeya dianthicola*. Appl. Environ. Microbiol. 82:268–278.

Felcher, K. J., Coombs, J. J., Massa, A. N., Hansey, C. N., Hamilton, J. P., and Veilleux, R. E. 2012. Integration of two diploid potato linkage maps with the potato genome sequence. PLoS One. 7:e36347

Friedman, M., Roitman, J. N., and Kozukue, N. 2003. Glycoalkaloid and calystegine contents of eight potato cultivars. J. Agric. Food Chem. 51:2964–2973.

Frost, K. E., Groves, R. L., and Charkowski, A. O. 2013. Integrated control of potato pathogens through seed potato certification and provision of clean seed potatoes. Plant Dis. 97:1268–1280.

Gomes, F. I. A., Teixeira, P., Azeredo, J., and Oliveira, R. 2009. Effect of farnesol on planktonic

Górniak, I., Bartoszewski, R., and Króliczewski, J. 2019. Comprehensive review of antimicrobial activities of plant flavonoids. Phytochem Rev. 18:241–272.

Gutiérrez-Barranquero, J. A., Reen, F. J., McCarthy, R. R., and O'Gara, F. 2015. Deciphering the role of coumarin as a novel quorum sensing inhibitor suppressing virulence phenotypes in bacterial pathogens. Appl. Microbiol. Biotechnol. 99:3303–3316.

Hao, J., Jiang, H., and Johnson, S. 2016. Detection and characterization of Dickeya species in the outbreak of blackleg disease of potato in Maine. Phytopathology. 106:2–2.

Hooker WJ, 1981. Compendium of potato diseases. St Paul, MN, USA: APS Press.

HosakaK. and HannemanR.E. 1998. Genetics of self-compatibility in a self-incompatible wild diploid potato species *Solanum chacoense*. 1. Detection of an S locus inhibitor (Sli) gene. Euphytica. 99: 191– 197.

Husain, F. M., Ahmad, I., Khan, Mohammad S., Ahmad, E., Tahseen, Q., Khan, Mohd Shahnawaz, et al. 2015. Sub-MICs of Mentha piperita essential oil and menthol inhibits AHL mediated quorum sensing and biofilm of Gram-negative bacteria. Front. Microbiol. 6:240.

Hussein, M., Han, M.-L., Zhu, Y., Zhou, Q., Lin, Y.-W., Hancock, R. E. W., et al. 2019. Metabolomics study of the synergistic killing of polymyxin B in combination with amikacin against polymyxin-susceptible and resistant *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 64:e01587-19

Jansky, S. H., and Peloquin, S. J. 2006. Advantages of wild diploid *Solanum* species over cultivated diploid relatives in potato breeding programs. Genet. Resour. Crop. Evol. 53:669–674.

Jansky, S. H., Chung, Y. S., and Kittipadukal, P. 2014. M6: A diploid potato inbred line for use in breeding and genetics research. J. Plant Regist. 8:195–199.

Ji, G., Beavis, R. C., and Novick, R. P. 1995. Cell density control of *Staphylococcal* virulence mediated by an octapeptide pheromone. PNAS. 92:12055–12059..

Jiang, H. H., Hao, J. J., Johnson, S. B., Brueggeman, R. S., and Secor, G. 2016. First report of *Dickeya dianthicola* causing blackleg and bacterial soft rot on Potato in Maine. Plant Dis. 100:2320–2320.

Johnson, T. L., Abendroth, J., Hol, W. G., and Sandkvist, M. 2006. Type II secretion: from structure to function. FEMS Microbiol. Lett. 255:175–186.

Joshi, J. R., Burdman, S., Lipsky, A., and Yedidia, I. 2015. Effects of plant antimicrobial phenolic compounds on virulence of the genus *Pectobacterium*. Res. Microbiol. 166:535-545.

Joshi, J. R., Burdman, S., Lipsky, A., Yariv, S., and Yedidia, I. 2016. Plant phenolic acids affect the virulence of *Pectobacterium aroidearum* and *P. carotovorum* spp. *brasiliense* via quorum sensing regulation. Mol. Plant Pathol. 17:487-500.

Joshi, J. R., Yao, L., Charkowski, A. O., and Heuberger, A. L. 2020. Metabolites from wild potato inhibit virulence factors of the soft rot and blackleg pathogen *Pectobacterium brasiliense*. MPMI. 34:100-109.

Jung, J.-E., Pandit, S., and Jeon, J.-G. 2014. Identification of linoleic acid, a main component of the n-hexane fraction from *Dryopteris crassirhizoma*, as an anti-*Streptococcus* mutans biofilm agent. Biofouling. 30:789–798.

Justice, S. S., Hunstad, D. A., Cegelski, L., and Hultgren, S. J. 2008. Morphological plasticity as a bacterial survival strategy. Nat. Rev. Microbiol. 6:162–168.

Kanoh, S., and Rubin, B. K. 2010. Mechanisms of action and clinical application of macrolides as immunomodulatory medications. Clin. Microbiol. Rev. 23:590–615.

Karim, S., McNally, R. R., Nasaruddin, A. S., DeReeper, A., Mauleon, R. P., Charkowski, A. O., et al. 2019. Development of the automated primer design workflow uniqprimer and diagnostic primers for the broad-host-range plant pathogen *Dickeya dianthicola*. Plant Dis. 103:2893–2902.

Kaufmann, G. F., Park, J., and Janda, K. D. 2008. Bacterial quorum sensing: a new target for anti-infective immunotherapy. Expert Opin Biol Ther. 8:719–724.

Konno, C., Lu, Z.-Z., Xue, H.-Z., Erdelmeier, C. A. J., Meksuriyen, D., Che, C.-T., et al. 1990. Furanoid lignans from *Larrea tridentata*. J. Nat. Prod. 53:396–406.

Kowalczyk, A., Eyice, Ö., Schäfer, H., Price, O. R., Finnegan, C. J., Egmond, R. A. van, et al. 2015. Characterization of para-nitrophenol-degrading bacterial communities in river water by using functional markers and stable isotope probing. Appl. Environ. Microbiol. 81:6890–6900.

Lai, Z., Tsugawa, H., Wohlgemuth, G., Mehta, S., Mueller, M., Zheng, Y., Ogiwara, A., Meissen, J., et al. 2018. Identifying metabolites by integrating metabolome databases with mass spectrometry cheminformatics. Nat. Methods. 15:53-56.

Lee, J.-H., Kim, Y.-G., Cho, M. H., Kim, J.-A., and Lee, J. 2012. 7-fluoroindole as an antivirulence compound against *Pseudomonas aeruginosa*. FEMS Microbiol. Lett. 329:36–44.

Leisner, C. P., Hamilton, J. P., Crisovan, E., Manrique-Carpintero, N. C., Marand, A. P., Newton, L., et al. 2018. Genome sequence of M6, a diploid inbred clone of the high-glycoalkaloid-producing tuber-bearing potato species Solanum chacoense, reveals residual heterozygosity. Plant J. 94:562–570.

Li, Y., Hutchins, W., Wu, X., Liang, C., Zhang, C., Yuan, X., et al. 2014. Derivative of plant phenolic compound inhibits the type III secretion system of *Dickeya dadantii* via HrpX/HrpY two-component signal transduction and Rsm systems. Mol. Plant Pathol. 16:150–163.

Li, Y., Peng, Q., Selimi, D., Wang, Q., Charkowski, A. O., Chen, X., et al. 2009. The plant phenolic compound p-coumaric acid represses gene expression in the *Dickeya dadantii* Type III secretion system. Appl. Environ. Microbiol. 75:1223–1228.

Lin, P.-Y., Yeh, K.-S., Su, C.-L., Sheu, S.-Y., Chen, T., Ou, K.-L., et al. 2012. Synthesis and antibacterial activities of novel 4-hydroxy-7-hydroxy- and 3-carboxycoumarin derivatives. Molecules. 17:10846–10863.

Lindhout, P., Meijer, D., Schotte, T., Hutten, R. C. B., Visser, R. G. F., and van Eck, H. J. 2011. Towards F1 Hybrid Seed Potato Breeding. Potato Res. 54: 301-312.

Little, T. J., Shuker, D. M., Colegrave, N., Day, T., and Graham, A. L. 2010. The Coevolution of virulence: tolerance in perspective ed. Marianne Manchester. PLoS Pathog. 6:e1001006

LittleT. 1945. Gene segregation in autotetraploids. Bot. Rev. 11: 60– 85

LittleT.M. 1952. Gene segregation in autotetraploids II. Bot. Rev. 24: 318– 339.

Liu, H., Coulthurst, S. J., Pritchard, L., Hedley, P. E., Ravensdale, M., Humphris, S., Burr, et al. 2008. Quorum sensing coordinates brute force and stealth modes of infection in the plant pathogen *Pectobacterium atrosepticum*. PLoS Pathog. 4:e1000093.

Liu, M.-C., Lin, S.-B., Chien, H.-F., Wang, W.-B., Yuan, Y.-H., Hsueh, P.-R., et al. 2012. 10′(Z),13′(E)-Heptadecadienylhydroquinone inhibits swarming and virulence factors and increases polymyxin B susceptibility in Proteus mirabilis. PLoS One. doi: 10.1371/journal.pone.0045563

Liu, M.-C., Lin, S.-B., Chien, H.-F., Wang, W.-B., Yuan, Y.-H., Hsueh, P.-R., et al. 2012. 10′(Z),13′(E)-Heptadecadienylhydroquinone inhibits swarming and virulence factors and increases polymyxin B susceptibility in *Proteus mirabilis*. PLoS One. 7:e45563

Lou, Z., Wang, H., Rao, S., Sun, J., Ma, C., and Li, J. 2012. *p*-Coumaric acid kills bacteria through dual damage mechanisms. Food Control. 25: 550-554.

Ma, B., Hibbing, M. E., Kim, H.-S., Reedy, R. M., Yedidia, I., Breuer, Jane, et al. 2007. Host range and molecular phylogenies of the soft rot enterobacterial genera *Pectobacterium* and *Dickeya*. Phytopathol. 97:1150–1163.

Magesh, H., Kumar, A., Alam, A., Priyam, null, Sekar, U., Sumantran, V. N., et al. 2013. Identification of natural compounds which inhibit biofilm formation in clinical isolates of *Klebsiella pneumoniae*. Indian J. Exp. Biol. 51:764–772.

Mahizan, N. A., Yang, S.-K., Moo, C.-L., Song, A. A.-L., Chong, C.-M., Chong, C.-W. 2019. Terpene derivatives as a potential agent antimicrobial resistance (AMR) pathogens. Molecules. 24: 2631.

McKnight, S. L., Iglewski, B. H., and Pesci, E. C. 2000. The *Pseudomonas* quinolone signal regulates *rhl* quorum sensing in *Pseudomonas aeruginosa*. J. Bacteriol. 182:2702–2708.

Mitchell, G., Lafrance, M., Boulanger, S., Séguin, D. L., Guay, I., Gattuso, M., et al. 2012. Tomatidine acts in synergy with aminoglycoside antibiotics against multiresistant *Staphylococcus aureus* and prevents virulence gene expression. J. Antimicrob. Chemother. 67:559–568.

Molodykh, Zh. V., Anisimova, N. N., Kudrina, M. A., Loseva, M. S., Nikonov, G. N., and Erastov, O. A. 1983. Antimicrobial activity of certain derivatives of phosphine and phenylphosphine. Pharm. Chem. J. 17:206–210.

Monte, J., Abreu, A. C., Borges, A., Simões, L. C., and Simões, M. 2014. Antimicrobial activity of selected phytochemicals against *Escherichia coli* and *Staphylococcus aureus* and their biofilms. Pathogens. 3:473–498.

Mun˜oz-Cazares, N., Garc´ıa-Contreras, R., Perez- L ´ opez, M., and Castillo- ´ Juarez, I. 2017. Phenolic compounds with anti-virulence properties. Intech. doi:10.5772/66367

Nasaruddin, A. S., Babler, B., Perna, N., Glasner, J., and Charkowski, A. 2019. First report of *Dickeya dianthicola* causing blackleg on Potato in Texas. Plant Dis. 103.

Nolte, P., Whitworth, J. L., Thornton, M. K., and McIntosh, C. S. 2004. Effect of seedborne Potato virus Y on performance of russet burbank, russet norkotah, and shepody potato. Plant Dis. 88:248–252.

Othman, L., Sleiman, A., and Abdel-Massih, R. M. 2019. Antimicrobial activity of polyphenols and alkaloids in middle eastern plants. Front. Microbiol. 10:911.

Panda, P., Vanga, B. R., Lu, A., Fiers, M., Fineran, P. C., Butler, R., Armstrong, et al. 2016. *Pectobacterium atrosepticum* and *Pectobacterium carotovorum* harbor distinct, independently

acquired integrative and conjugative elements encoding coronafacic acid that enhance virulence on potato stems. Front. microbiol. 7:397.

Parai, D., Banerjee, M., Dey, P., Chakraborty, A., Islam, E., and Mukherjee, S. 2018. Effect of reserpine on *Pseudomonas aeruginosa* quorum sensing mediated virulence factors and biofilm formation. Biofouling. 34:1–15.

Pédron, J., Mondy, S., Raoul des Essarts, Y., Van Gijsegem, F., and Faure, D. 2014. Genomic and metabolic comparison with *Dickeya dadantii* 3937 reveals the emerging *Dickeya solani* potato pathogen to display distinctive metabolic activities and T5SS/T6SS-related toxin repertoire. BMC Genomics. 15:283.

Pérombelon, M. C. M. 2002. Potato diseases caused by soft rot erwinias: an overview of pathogenesis. Plant Pathol. 51:1–12.

Qiu, J., Luo, M., Dong, J., Wang, J., Li, H., Wang, X., et al. 2011. Menthol diminishes *Staphylococcus aureus* virulence-associated extracellular proteins expression. Appl. Microbiol. Biotechnol. 90:705–712.

Qiu, J., Niu, X., Wang, J., Xing, Y., Leng, B., Dong, J., et al. 2012. Capsaicin Protects Mice from Community-Associated Methicillin-Resistant *Staphylococcus aureus* Pneumonia. PLOS ONE. 7:e33032.

Qiu, J., Wang, D., Xiang, H., Feng, H., Jiang, Y., Xia, L., et al. 2010. Subinhibitory concentrations of thymol reduce wnterotoxins A and B and α-hemolysin production in *Staphylococcus aureus* isolates. PLOS ONE. 5:e9736

R Core Team. 2015. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Råberg, L. 2014. How to Live with the Enemy: Understanding Tolerance to Parasites. PLoS Biol. 12:e1001989

Ragunath, C., Shanmugam, M., Bendaoud, M., Kaplan, J. B., and Ramasubbu, N. 2012. Effect of a biofilm-degrading enzyme from an oral pathogen in transgenic tobacco on the pathogenicity of *Pectobacterium carotovorum* subsp. *carotovorum*. Plant Pathol. 61:346–354.

Redondo, L. M., Chacana, P. A., Dominguez, J. E., and Miyakawa, M. E. F. 2014. Perspectives in the use of tannins as alternative to antimicrobial growth promoter factors in poultry. Front. Microbiol. 5:118

Reen, F. J., Gutiérrez-Barranquero, J. A., Parages, M. L., and O´Gara, F. 2018. Coumarin: a novel player in microbial quorum sensing and biofilm formation inhibition. Appl. Microbiol. Biotechnol. 102:2063.

Reverchon, S., and Nasser, W. 2013. *Dickeya* ecology, environment sensing and regulation of virulence programme. Environ. Microbiol. Rep. 5:622–636.

Rukayadi, Y., and Hwang, J.-K. 2006. Effect of coating the wells of a polystyrene microtiter plate with xanthorrhizol on the biofilm formation of *Streptococcus* mutans.J. Basic. Microbiol. 46:410–415.

Rutherford, S. T., and Bassler, B. L. 2012. Bacterial quorum sensing: its role in virulence and possibilities for its control. Cold Spring Harb. Perspect. Med. 13:a012427

Sadowska, B., Kuźma, Ł., Micota, B., Budzyńska, A., Wysokińska, H., Kłys, A., et al. 2016. New biological potential of abietane diterpenoids isolated from Salvia austriaca against microbial virulence factors. Micron. Pathog. 98.132-139.

Shryock, T., Mortensen, J., and Baumholtz, M. 1998. The effects of macrolides on the expression of bacterial virulence mechanisms. J. Antimicron. Chemother. 41:505–12.

Smith, C. A., Want, E. J., O'Maille, G., Abagyan, R., and Siuzdak, G. 2006. XCMS: Processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. Anal. Chem. 78:779-787.

Smith, R. S., and Iglewski, B. H. 2003. P. aeruginosa quorum-sensing systems and virulence. Curr. Opin. Microbiol. 6:56–60.

Strauss, S. Y., and Agrawal, A. A. 1999. The ecology and evolution of plant tolerance to herbivory. Trends Ecol. Evol. 14:179–185.

Tao, R., Wang, C., Ye, J., Zhou, H., and Chen, H. 2016. Polyprenols of Ginkgo biloba enhance antibacterial activity of five classes of antibiotics. Biomed Res. Int. 2016:1–8.

Taylor, D., Charkowski, A. O., and Zeng, Y. 2020. Laboratory assays used to rank potato cultivar tolerance to blackleg showed that tuber vacuum infiltration results correlate with field observations. Plant Dis. doi:10.1094/PDIS-07-20-1485-RE

Tomiyama, K., Mukai, Y., Saito, M., Watanabe, K., Kumada, H., Nihei, T., et al. 2016. Antibacterial action of a condensed tannin extracted from astringent persimmon as a component of food addictive aancil PS-M on oral polymicrobial biofilms. BioMed Research International. 2016:5730748

Torres-Romero, D., Jiménez, I. A., Rojas, R., Gilman, R. H., López, M., and Bazzocchi, I. L. 2011. Dihydro-β-agarofuran sesquiterpenes isolated from *Celastrus vulcanicola* as potential anti-*Mycobacterium tuberculosis* multidrug-resistant agents. Bioorg. Med. Chem. 19:2182–2189.

Truchado, P., Tomás-Barberán, F. A., Larrosa, M., and Allende, A. 2012. Food phytochemicals act as Quorum sensing inhibitors reducing production and/or degrading autoinducers of *Yersinia enterocolitica* and *Erwinia carotovora*. 24:78-85

Tsugawa, H., Kind, T., Nakabayashi, R., Yukihira, D., Tanaka, W., Cajka, T., Saito, K., Fiehn, O., and Arita, M. 2016. Hydrogen rearrangement rules: Computational MS/MS fragmentation and structure elucidation using MS-FINDER software. Anal. Chem. 88:7946-7958.

Vijayaraghavan, P., and Vincent, S. G. P. 2013. A simple method for the detection of protease activity on agar plates using bromocresolgreen dye. J. Biochem. Technol. 4:628-630.

Visser, R. G. F., Bachem, C. W. B., Boer, J. M. de, Bryan, G. J., Chakrabati, S. K., Feingold, S., et al. 2009. Sequencing the potato genome: outline and first results to come from the elucidation of the sequence of the world's third most important food crop. Am. Potato. J. 86: 417–429.

Walsh, D. J., Livinghouse, T., Goeres, D. M., Mettler, M., and Stewart, P. S. 2019. Antimicrobial activity of naturally occurring phenols and derivatives against biofilm and planktonic bacteria. Front Chem. 7:653.

Wang, S., Alseekh, S., Fernie, A. R., and Luo, J. 2019. The structure and function of major plant metabolite modifications. Mol. Plant 12: 899-919.

Xu, X., Pan, S., Cheng, S., Zhang, B., Mu, D., Mu, D., et al. 2011. Genome sequence and analysis of the tuber crop potato. Nature. 475:189–195.

Yang, L., Ding, W., Xu, Y., Wu, D., Li, S., Chen, J., et al. 2016. New insights into the antibacterial activity of hydroxycoumarins against *Ralstonia solanacearum*. Molecules. 21.

Yogendra, K. N., Kushalappa, A. C., Sarmiento, F., Rodriguez, E., and Mosquera, T. 2014. Metabolomics deciphers quantitative resistance mechanisms in diploid potato clones against late blight. Funct. Plant Biol. 43:284-298

Yoon, B. K., Jackman, J. A., Valle-Gonzalez, E. R., and Cho, N.-J. 2018. Antibacterial free fatty acids and monoglycerides: Biological activities, experimental testing, and therapeutic applications. Int. J. Mol. Sci. 19:1114.

Zeng, Y., and Charkowski, A. O. 2020. The role of ATP-binding cassette (ABC) transporters in bacterial phytopathogenesis. Phytopathol. doi:10.1094/PHYTO-06-20-0212-RVW

Zeng, Y., Fulladolsa, A. C., Houser, A., and Charkowski, A. O. 2019. Colorado seed potato certification data analysis shows mosaic and blackleg are major diseases of seed potato and identifies tolerant potato varieties. Plant Dis. 103:192–199.

Zimnoch-Guzowska, E., Marczewski, W., Lebecka, R., Flis, B., Salamini, F., and Gebhardt, C. 2000. QTL analysis of new sources of resistance to *Erwinia carotovora* spp. *atrosepticum* in potato done by AFLP, RFLP, and resistance-gene-like markers. Crop Sci. 40:1156-1167.

CHAPTER V: SUMMARY

Rapid increase in population and food demand has placed immense pressure on agriculture and natural resources. By 2050, a global population of 9.7 billion people will demand 70% more food than is consumed today, but water and soil resources will be more limited than they are today, and climate change will put increasing pressure on food production. Therefore, providing sufficient food requires sustainable and substantial improvement in global food production, but it will only succeed if we use methods that do not further increase environmental degradation and further accelerate climate change.

Potato is one of the leading crops grown in worldwide and is the fourth most important staple food source after wheat, rice, and corn. Compared to other major crops, potato is also one of the most water-efficient crops and one of the most versatile crops. For example, potato is grown in high deserts, lowland tropics, and near the arctic circle. In the United States, potato is the leading vegetable crop consumed and commercially grown in 30 states, including Colorado, with a total production value of approximately $4 billion annually. However, this important crop that is a potential future food source is affected by about 160 different diseases and disorders including over 50 fungal, 10 bacterial, and 40 viral diseases. Each year, approximately $40 million in losses are caused by the blackleg and soft rot diseases in the US.

My first research objective was to develop tools to improve current *Dickeya dianthicola* and *Clavibacter michiganenesis* subsp. *sepedonicus* (Cms) detection methods, which will (describe here). My second objective was to identify molecules associated with resistance of blackleg disease, which will improve current management strategies and hence strengthen the foundations for future breeding programs to improve crop resistance against blackleg and soft rot

diseases of potato. My research has contributed to food security and sustainability by developing accessible and robust detection tools and by demonstrating that blackleg resistant plants can be quickly identified through simple biochemical assays. I also made progress in identification of metabolites that may contribute to blackleg disease resistance. Overall, my research contributed to three areas of disease management: detection, screening for resistance, and in breeding program.

The blackleg pathogens *Dickeya* and *Pectobacterium* are highly variabile, which has made development of detection assays difficult. Almost all published assays have failed because either the assay is not specific enough and detects species in addition to the target pathogen, or it is too specific and does not detect all strains within the target species. For example, the PelADE assay (reference) detects bacterial species in addition to *Dickeya* and the DiaA assay, which targets *D. dianthicola*, does not detect all strains present in the United States.

I tested a user-friendly online computer program, Uniqprimer to determine if it could be used to develop useful detection assays for *D. dianthicola* (REF). This computer program can use bacterial genome sequences to quickly and automatically design polymerase chain reaction (PCR) assays for pathogens. Uniqprimer will have a high impact on agriculture because this program can quickly design PCR tests, it is freely available to anyone, and it requires only basic computer operating skills. Plant pathologist have already started using Uniqprimer to develop PCR tests for other blackleg associated pathogen such as *Pectobacterium* strains, which is common pathogen in North America. For example, the plant diagnostic clinic at CSU is validating a PCR tested for *Pectobacterium parmentieri*, an aggressive soft rot pathogen that is often found alongside *Dickeya* spp. in potatoes.

In addition, we also developed a droplet digital PCR (ddPCR) assay to detect a quarantine pathogen called *Clavibacter michiganenesis* subsp. *sepedonicus*. ddPCR is an advanced detection

tool but not yet available to every plant diagnostic facility, however, the rapid advances in technology nowadays, the tools necessary for accurate detection will be accessible to all diagnostic facilities soon. We empirically compared the ddPCR assay to the existing detection methods including conventional PCR, real-time PCR, and enzyme-linked immunosorbent assay (ELISA). The ddPCR assay has improved detection by 100-fold compared to qPCR and therefore a robust detection tool compared to other conventional detection methods.

These detection assays will help to mitigate yield losses by accurately detecting disease causing pathogens and will help researchers in determining how these pathogens enter into seed potato systems and spread among the farms. With development of these assays, the U.S growers will be able to use tests to more accurately determine the species causing blackleg and soft rot on their farms, which is the first step in finally being able to effectively manage this common scourge of potato production. Notably, the method used to develop these detection tools are applicable to any plant pathogen anywhere in the world.

Scientists lack information on the relative resistance of potato varieties to *Dickeya* spp. This information is crucial for breeding programs and epidemiological or evolutionary studies, and hence disease management. Moreover, both potato breeders and farmers have insufficient knowledge of molecular tools to identify potato lines that are likely to be resistant or tolerant to blackleg or soft rot diseases of potato.

In effort to increase the breeding tools available for resistance to blackleg disease, we identified two resistant and two susceptible accessions of wild diploid potato species (*S. chacoense*) using physiological, biochemical and metabolic profiles. We found that our biochemical assays were highly correlated with metabolic profile of resistant plants. For example, we detected metabolites associated with inhibition of quorum sensing (QS) in metabolic profile of

resistant stem extracts. We found similar results in our lab experiment, where resistant stem extracts caused reduced activity of plant cell wall degrading enzymes and QS activity in the blackleg pathogens. Metabolic profile of resistant accessions reveled that antimicrobial and anti-virulence properties of these metabolites may contribute to blackleg and soft rot resistance by inhibition of QS activity and production of plant cell wall degrading enzymes (PCWDEs).

We anticipate that the plant resistance in potatoes against soft rot *Pectobacteriaceae* (SRP) may triggered by QS inhibiting metabolites, which in addition suppresses PCWDEs and biofilm formation and hence attenuate disease. I believe there are still more to explore in the same thread of information and should be investigated further. Some of the most important questions are:

1. Are the QS inhibiting molecules the primary mode of resistant in plants against SRP in potato and in other plants?
2. What genes and pathways are required for production of these metabolites?
3. How did these genes evolve?
4. How is production of these metabolites regulated?
5. Does the plant microbiome affect production of these plant metabolites?
6. Do the microbiomes of resistant and susceptible plants differ due to production of the SRP-inhibiting metabolites?

Interestingly, some studies have reported the soil microbiome as one of the major drivers of plant responses against biotic and abiotic stresses. Further investigation on the role of soil microbe in resistance plants could be very useful to understand host-microbe interactions. It will add crucial information to the disease triangle and help us to make one step forward in the development of cultivars with more durable resistance to the diseases caused by pathogens.